UNIVERSITY OF ALBERTA

IN VITRO PRODUCTION AND LONG TERM STORAGE OF MAIZE ZYGOTIC EMBRYOS DERIVED FROM ISOLATED EMBRYO SACS

by

JOHN DRUMMOND LAURIE ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE

DEPARTMENT OF BIOLOGICAL SCIENCES

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled *IN VITRO* PRODUCTION AND LONG TERM STORAGE OF MAIZE ZYGOTIC EMBRYOS DERIVED FROM ISOLATED EMBRYO SACS submitted by JOHN D. LAURIE in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE.

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James D. Young

Date: June 20, 1997

Science is built up with facts, as a house is with stones.
But a collection of facts is no more a science than
a heap of stones is a house.

Jules Henri Poincaré

DEDICATION

To my parents Janet and John

ABSTRACT

A novel embryo sac isolation method was developed employing mechanical sectioning of maize ovaries using the Vibratome. Isolated sections containing intact embryo sacs were viable and developed *in vitro* on MS media producing endosperm (90%) and embryos (75%). Embryos (10-14 DAP) were frozen to -196°C in liquid nitrogen (LN). Root and shoot apices exhibited differential sensitivity to cooling rate. To overcome this problem embryos were vitrified in VSY2 (10% propylene glycol, 15.5% ethylene glycol, 20.5% dimethyl sulfoxide, 6M polyethylene glycol). After a 48 hour incubation on MS containing 15% sucrose and 50µM abscisic acid (ABA) to increase desiccation tolerance 46% of the embryos survived vitrification, storage in LN and warming. This method was then applied to *in vitro* produced embryos derived from 1 DAP embryo sacs. Survival (82%) was higher for these embryos indicating a greater degree of desiccation tolerance. Immediate applications are the use of isolated embryo sacs as targets for genetic manipulation, and vitrification as a means to preserve transgenic embryos.

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LIST OF ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
ABA	abscisic acid
BAP	6-benzylaminopurine
DAP	days after pollination
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
FAA	5% formalin, 5% acetic acid, and 45% ethanol in water
FDA	fluorescein diacetate
LN	liquid nitrogen
MS	Murashige and Skoog medium
NLS	nuclear localization signal
PBS	phosphate-buffered saline
PVS2	vitrification solution
T-DNA	transfer DNA
TOWILL	vitrification solution
TTC	2,3,5-triphenyltetrazolium chloride
VirD2	*Agrobacterium* virulence protein D2
VirE2	*Agrobacterium* virulence protein E2
VSY2	vitrification solution

CHAPTER 1

GENERAL INTRODUCTION

Fertilization followed by cell division and differentiation from zygotes to embryos are critical events in the life cycle of plants and have been the subject of numerous scientific investigations (Breton et al., 1995; Russell, 1993). Gregor Mendel through plant breeding was able to identify discrete inheritable characteristics or genes. But even before Mendel humans have been fascinated with plant sexual reproduction. For thousands of years we have used basic knowledge of pollination and seed development for selective breeding resulting in the domestication of numerous plant species. With the invention of the microscope and development of histological techniques for preserving and sectioning biological material, our knowledge of plant sexual reproduction increased to the cellular level. Modern histological studies revealed the overall anatomy identifying cells that play key roles in plant pollination and fertilization, but molecular and biochemical studies lagged (Mol et al., 1995). The reason is that the megagametophyte or embryo sac is deeply enclosed in maternal sporophytic tissue. Gaining access to viable embryo sacs therefore was a limiting factor until recently. Surgical and enzymatic digestion methods for isolating embryo sacs have recently permitted *in vitro* fertilization and the development of protocols for regeneration of plants from zygotes (Kovac et al., 1995; Kranz and Lorz, 1993). These studies are still, however, in their infancy since fusion of gametes is difficult and not well understood (Russell, 1993; Tirlapur et al., 1995).

Interest in embryogenesis increased significantly with the development of methods to generate embryos from somatic cells and from microspores (Bajaj, 1990; Emons, 1994). In addition the previous recalcitrant nature of cereal crops to agroinfection directed attention toward embryogenic cells as targets for genetic engineering (Songstad et al., 1995; Vasil, 1988). Direct introduction of transgenes into embryogenic cells followed by the induction of embryogenesis has produced transgenic plants (Songstad et al., 1995). Most of these direct

genetic transformation methods, however, involved callus-based selection protocols and extensive *in vitro* procedures which are both time consuming and can yield somaclonal variants (Devemo, 1995; Phillips et al., 1994). With the development of *in vitro* fertilization and relatively simple procedures to regenerate plants directly from zygotes there was a renewed interest in zygotic embryogenesis. Transformation of zygotes and direct embryogenesis circumvent methods leading to chimeric plants with somaclonal alterations (Leduc et al., 1996). With basic research focused on understanding embryogenesis and applied research focused on exploiting embryogenic tissues for commercial purposes, knowledge of embryogenesis increased exponentially. This interest in zygotic embryogenesis created a demand for improved gamete isolation and simplified plant regeneration procedures. The focus of this thesis is on zygotic embryogenesis, cryopreservation of embryos, and the use of a mechanical sectioning method to isolated embryo sacs containing zygotes.

Overview of the Reproduction Process in Angiosperms

In higher plants the sporophyte is the dominant life form, while the gametophyte is significantly reduced. In angiosperms gametogenesis is separated into microgametogenesis and megagametogenesis in specialized structures called flowers, resulting in pollen and embryo sacs respectively (Chang and Neuffer, 1989; Huang and Russell, 1992). Depending on the species, flowers are bisexual or unisexual. In maize, flowers are unisexual and plants are monoecious. Male flowers are grouped together in an apical inflorescence (tassel), while female flowers are grouped into medial inflorescences (cobs or ears). The diploid sporophyte undergoes meiosis in both the cobs and tassels initially resulting in megaspores and microspores respectively. In maize three of the four megaspores usually degrade (Russell, 1979). Mitotic divisions of the microspores and megaspores cells during gametogenesis leads to mature pollen and embryo sacs. Mature maize pollen grains contain two sperm cells within a vegetative cell all surrounded by a thick

wall. Pollen grains are released from anthers during anthesis, and carried by wind to cobs. Mature embryo sacs consist of at least seven discrete cells. Near the micropyle is the egg apparatus containing an egg cell and two synergids. Centrally located in embryo sacs is the central cell containing two polar nuclei positioned adjacent to the egg apparatus. Within the central cell is at least one large vacuole, and towards the chalaza are antipodal cells, which are numerous in maize. Embryo sacs are surrounded by nucellus which is enclosed by two integuments forming the ovule. Ovules are protected by an ovary wall enclosed by two layers of perianth consisting of the outer lemma, inner lemma, outer glume and inner glume. It is this combined protection of perianth, ovary wall, integuments, and nucellus that make viable embryo sacs difficult to isolate.

Pollination occurs when pollen grains land on silks and germinate to produce pollen tubes. These tubes travel down the silk toward the ovule where one tube will enter the embryo sac via the micropyle. The pollen tube will penetrate and release two sperms into one synergid which has degenerated. Sperms subsequently fuse independently with the egg and central cell resulting in double fertilization producing the zygote and endosperm respectively. After a period of coenocytic divisions endosperm becomes cellular and continues to divide before storing reserves prior to maturation drying. Division of the zygote initially yields a suspensor and the embryo proper which continues to develop into an embryo. By approximately 10 days after pollination (DAP) maize embryos have developed scutella and root and shoot apices (Van Lammeren, 1987). As maturation proceeds storage reserves are deposited and embryos begin to dry. The ovule forms a seed which is desiccated (<9% water) and can remain viable for a long time before rehydration (imbibition) and germination (Williams and Leopold, 1989).

Embryo Sac Isolation

Embryo sacs have been isolated from ovules to different using either digestive enzymes or microdissection in various combinations (Wagner et al.,

1989). Digestive enzymes are used to weaken cell walls so that nucellar cells break away and expose embryo sacs, resulting in completely isolated embryo sacs. Access to embryo sacs using this approach, unfortunately, results in disruption of cell walls within embryo sacs as well, which may adversely effect embryo sac viability (Leduc et al., 1995). In addition, the nucellar cells removed during isolation may be important for initiating and controlling the early stages of embryogenesis (Campenot et al., 1992). Microdissection on the other hand, involves physically cutting nucellus away from embryo sacs. This can be labor intensive, but enzymes are avoided thus leaving embryo sacs intact. As well, it is virtually impossible to remove all of the nucellus from the embryo sac, and therefore visual access to individual embryo sac cells is often hampered. For embryogenesis studies, however, the nucellus that remains after microdissection may be beneficial to development. Choosing the appropriate isolation method therefore depends on the application and the requirement for embryo sac visibility.

Applications of Isolated Embryo Sacs

Isolation of viable (living) embryo sacs is of considerable interest. The potential for studying fertilization, embryogenesis and endosperm development is immense. *In vivo* as well as *in vitro* fertilization can be studied in living embryo sacs (Kranz and Lorz, 1993). This approach creates the potential for selection of desired embryo sacs and sperm cells and provides the ability to manipulate the *in vitro* environment during fertilization. Crosses not occurring naturally due to incompatibility could result in creation of new crops. As well, transgenic sperm or embryo sacs can be used for *in vitro* fertilization procedures to produce transgenic plants. Similarly transgenes could be introduced into fertilized embryos sacs which could develop into transgenic crops (Leduc et al., 1996). Embryogenesis and endosperm development can be studied from fertilization providing information on the early stages of development. These stages can be studied with both the nucellus intact or with it removed, which may provide a

means in which the role of nucellus in stimulating embryogenesis can be studied. Cellular as well as genetic and biochemical information can be obtained. As well, manipulation of embryo sacs during these early stages may reveal genes that are important and exclusive to these stages. Manipulation of these genes may further our understanding of seed development and lead to crop improvement.

Overview of Maize *In Vitro* Biology

The totipotent nature of many plant somatic cells and their ability to survive *in vitro* has stimulated enormous interest and research into plant *in vitro* biology. Early studies focused on developing appropriate media to support growth and development of plant cells, but once *in vitro* conditions were in hand, emphasis soon turned to studying the behavior of cells. It was found that somatic cells often divide (dedifferentiate) *in vitro* into a mass of relatively undifferentiated cells called callus, and that organogenesis and embryogenesis sometimes occurred from these dedifferentiated cells. Hormonal balance was later determined to control the fate of cell differentiation (Skoog and Miller, 1957). Today explants come from a number of sources and are used for a variety of applications (Roest and Gilissen, 1993). In maize, embryogenic cultures are derived from immature embryos (Duncan et al., 1985). Different types of callus can be stimulated to undergo embryogenesis (Welter et al., 1995). More organized structures can also be cultured *in vitro*. For example, apical meristems are cultured *in vitro* and induced to form multiple shoots resulting in mass propagation (Finch et al., 1992). For other species, embryos derived from callus or cell cultures can mature *in vitro* and be stored in liquid nitrogen or dried and coated to produce artificial seeds (Attree and Fowke, 1993; Mycock et al., 1995). Application of these preservation techniques to maize *in vitro* procedures would therefore be desirable.

Cryopreservation

Cryopreservation is commonly used to store both plant and animal cells and tissues. It involves cooling to subzero temperatures resulting in arrest of metabolism and prevention of decay. Optimally cells should be stored below -100°C, the temperature below which biological functions cease, and that ice recrystallization is minimal (Mazur, 1970). Since most cells and tissues cannot be dried, cryostorage must be done in solution. When solutions are cooled below their freezing point ice forms which can be lethal (Karlsson et al., 1993). Ice nucleation in cell suspensions originates in the extracellular environment causing the unfrozen solution to become more concentrated. This change in osmotic potential results in cell dehydration. As temperature falls below the freezing point more and more ice forms further increasing extracellular concentration and cell dehydration. Ice formation becomes lethal when the cooling rate is too high for cells to respond osmotically resulting in intracellular freezing, or when ice-induced dehydration becomes lethal (Mazur, 1970). When freezing multicellular structures extracellular ice may become damaging when cellular connections are broken. Commonly cells are frozen using a method referred to as constant rate cooling. This method involves slow cooling to an intermediate subzero temperature (e.g. -40°C) followed by rapid cooling to -196°C by immersion in liquid nitrogen (Meryman and Williams, 1985). The initial step induces cell dehydration and during the second step rapid cooling promotes vitrification of the unfrozen cytoplasm (Rall, 1981). Stored cells therefore exist in a glassy state which is considered harmless. This same phenomenon is often used to store cells in the absence of ice formation (Fahy, 1988). Concentrated solutions are added to cell suspensions prior to rapid cooling. At sufficiently high cooling rates such solutions vitrify without forming ice. Cells that can withstand the initial dehydration caused during loading of cryoprotectants also survive vitrification.

Plant cryopreservation using various methods has been reported from protoplasts to complex, organized structures like embryos (Withers, 1980). Cell

suspensions and protoplasts were among the first to be cryopreserved, providing models of cell behavior at subzero temperatures (Langis et al., 1989). These early studies provided basic knowledge allowing cryopreservation to proceed to more complex structures like shoot tips and embryos (Mycock et al., 1995; Towill, 1990). Out of these studies have come numerous methods for getting plant cells to the temperature of liquid nitrogen. All involve dehydrating cells prior to cooling in order to avoid or reduce the harmful effects of ice formation. Consequently, numerous cryoprotective cocktails have been developed which remove water from cells and offer protection from dehydration. Wherever long term storage is required cryopreservation is becoming routine, especially in plant tissue culture laboratories.

Current developments in maize reproductive biology have created a need for improvements in gamete isolation and handling of embryogenic tissues (Leduc et al., 1995). Successful *in vitro* fertilization has sparked a renewed interest in zygotic embryogenesis (Kranz and Lorz, 1993). Difficulties, however, exist with current methods used to regenerate plants from zygotes. As well, all existing methods of gaining access to viable embryo sacs have disadvantages. The objective of this study is to overcome some of these difficulties by creating an alternative method of isolating maize embryo sacs and regenerating plants. As well, my goal is to provide a useful procedure for cryopreservation of maize immature embryos. The results of these studies will have a significant impact on maize reproductive biology. The mechanically isolated embryo sacs will provide basic knowledge about fertilization, embryogenesis, and endosperm development. Applications will be widespread including genetic transformation. Cryopreservation of maize immature embryos will be beneficial to tissue culture laboratories in which large numbers of embryos are handled.

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CHAPTER 2

A Novel Technique for the Isolation of Embryo Sacs from Maize and the Subsequent Regeneration of Plants

INTRODUCTION

Isolation of embryo sacs from protective maternal tissue is of considerable interest to flowering plant biologists. Since male gametes are easily accessible, isolation of the female gametophyte would allow *in vitro* fertilization and the detailed analysis of fertilization and development (Campenot et al., 1992). Gamete recognition, the blocking of polyspermy as well as the early stages of embryogenesis and endosperm development are examples of what could be studied. In addition, pre- and post-fertilization barriers may be avoided by *in vitro* fertilization of isolated gametes resulting in crosses not previously possible. Such crosses would result in a wider genetic base from which plant breeders could select for desirable traits. Crosses may not be necessary, however, with the techniques presently available to introduce foreign genetic material into plant cells (Songstad et al., 1995).

Isolated embryo sacs and sperm cells are suitable targets for genetic manipulation. Introduction of transgenes into one or both gametes before fertilization or into the vicinity of the egg and sperm cell at the time of fertilization may lead to stable transformation. As well, the introduction of transgenes into the zygote immediately following fertilization may also result in transformation (Leduc et al., 1996). All of these techniques are performed at or near the time of fertilization when only one or two cells are present, reducing the likelihood of producing chimeric plants. Microinjection, particle bombardment or *Agrobacterium tumefaciens* co-cultivation may also be used to introduce the transgenes into isolated embryo sacs (Songstad et al., 1995).

The isolation of embryo sacs from maize using digestive enzymes was first reported by Wagner et al. (1989). Since that time, egg protoplasts have been completely isolated and fused with isolated sperms by electrofusion (Kranz et al., 1991; Faure et al., 1994). Development and regeneration of plants from

these fusion products was accomplished using a feeder culture (Kranz and Lorz, 1993). As well, embryo sacs fertilized *in vivo* have been stimulated to develop *in vitro* leading to mature plants without the aid of feeder cells (Campenot et al., 1992; Mol et al., 1993). Recently *in vitro* fertilization was attempted by microinjecting sperm nuclei into embryo sacs of maize (Mathys-Rochon et al., 1994). These advances are creating a demand for easier and more rapid methods of obtaining embryo sacs and for improved *in vitro* regeneration procedures.

In this chapter I will discuss the advantages of isolating embryo sacs by mechanical sectioning using the Vibratome and make a comparison to other techniques. As well, the selection of an appropriate *in vitro* culture procedure for embryo sacs isolated using this method will be examined. The final part of this chapter will focus on the uses of isolated maize embryo sacs.

MATERIALS AND METHODS

Plant material

Plants of *Zea mays* L. cultivar CV129 were grown to maturity in growth rooms at the University of Alberta. At anthesis, pollen was collected by shaking tassels over an enamel tray and used immediately for hand pollination. Cobs were produced in a pollen-free environment by removing plants from the pollen-producing rooms prior to silk emergence. The plants were emasculated, washed free of pollen and placed in a pollen-free room for cob development. When emergent silks were 6 to 10 cm in length the plants were removed from the pollen-free room and hand pollinated with fresh pollen. The cobs were subsequently bagged and the plants then placed in a growth chamber for fertilization to occur (~16 hours for CV129). Embryo sacs were isolated at least 16 hours after pollination.

Embryo sac isolation

1. Sterilization of plant material: After removing the husks and silks, cobs were cut transversely into approximately 3 cm segments. The segments were surface sterilized for 10 minutes in 70% ethanol followed by three rinses in sterilized distilled, deionized water. Ovaries were then removed and mounted for sectioning.

2. Mounting: Specimen blocks were surface sterilized in 70% ethanol for 10 minutes. The blocks were then placed in a row in the laminar flow hood. After the alcohol had evaporated, a thin layer of fast acting adhesive (Quick Set 404, Locktite Corporation, Newington, CT) was applied to the top of each block and the ovaries were placed in the proper position. The ovaries were removed in pairs, with 2 or 3 pairs placed on each block. The ovaries were placed with their adaxial surface up, and perpendicular to the long axis of the specimen block (Fig. 2.1).

3. Sectioning: The blocks with the attached ovaries were placed in the Vibratome (Technical Products International, Inc., St. Louis, MO) with the stylar ends of the ovaries facing the blade (Fig. 2.2). The blade was positioned above the ovaries and the specimen block raised to begin sectioning. The ovaries were then sectioned at a thickness of 200 to 400 µm. The first block was serially sectioned at 200 µm increments from the adaxial surface of the ovaries. Every section was then observed to determine how far the embryo sac was from the surface. Once this was determined, sectioning commenced with the goal of slicing on either side of the embryo sacs. Thinner sections often resulted in better embryo sac transparency, but resulted in the loss of many embryo sacs. Thicker sections produced more intact embryo sacs, but the embryo sacs were often less transparent due to the thickness of the supporting nucellus. Sections 250 to 300 µm routinely produced numerous embryo sacs with acceptable

transparency. The sections containing the embryo sacs were collected and placed on semi-solid MS culture medium.

4. Handling sections: The sections were evaluated by observing both sides using a stereomicroscope with basal illumination. Sections were easily manipulated by grasping the ovary wall with fine forceps. Intact embryo sacs were placed with their more transparent side upward prior to microinjection and *in vitro* culture. The isolation procedure is shown in Figure 2.3.

Viability determination

The viability of isolated embryo sacs was determined by staining with fluorescein diacetate (FDA) or by culturing on modified Murashige and Skoog (MS) media according to Campenot et al. (1992). Sections containing embryo sacs were immersed in FDA for various lengths of time and then observed under blue light at 450-490nm. Unfortunately this method resulted in considerable fluorescence from surrounding cells, making observation of embryo sac fluorescence difficult. Alternatively, FDA was directly placed into the embryo sacs using microinjection, which resulted in far less background fluorescence. *In vitro* development was monitored daily using a Zeiss (Stemi SV11) stereophotomicroscope with basal illumination. Other embryo sacs were fixed in FAA (5% formalin, 5% acetic acid, and 45% ethanol in water) after 4 and 16 days in culture prior to embedment in Paraplast Plus and sectioning at 12-15μm thickness with a rotary microtome to observe any changes not detected using the stereomicroscope. The sections were stained with safranin and fast green and examined under bright-field using a Carl Zeiss photomicroscope.

In vitro culture of 1DAP embryo sacs

Embryo sacs grown *in vitro* on MS media were monitored for endosperm and embryo development. One DAP embryo sacs were placed on modified MS medium containing either high (15%) or low (6%) sucrose in the presence (0.1

mg/ml) or absence of the hormone 6-benzylaminopurine (BAP). Sections containing intact 1DAP embryo sacs were placed on each of the selected media and monitored daily. Triplicates of thirty embryos were used to calculate mean and standard error for each treatment.

RESULTS

The present study describes a method of isolating maize embryo sacs using mechanical live sectioning of ovaries. The method produced sections containing embryo sacs that were viable and could be manipulated and grown in culture. The isolation procedure was relatively inexpensive since it did not require digestive enzymes. As well, the embryo sacs were structurally intact and supported laterally by the nucellus, as opposed to embryo sacs isolated completely using digestive enzymes. The isolation procedure was relatively easy to perform and required far less skill than surgical microdissection. An experienced worker could isolate roughly 200 embryo sacs in a 3 hour period. Embryo sacs isolated using this method were easily manipulated and required less sophisticated *in vitro* culture to produce plants.

Partial isolation of maize embryo sacs was possible by serially sectioning live ovaries at a thickness of 250-300µm. Embryo sacs were approximately 400µm below the adaxial surface of the ovary wall (Campenot et al., 1992). The structural integrity of the embryo sacs was intact, since they were contained between thin layers of nucellus (Fig. 2.4A). The egg apparatus was visible showing distinctly the zygote (or egg) and one or two synergids depending upon the plane of the section. As well, the antipodal cells could be seen against the light background of nucellus. Sections containing the embryo sacs were handled with ease using forceps by grasping the ovary wall, and easily observed using a stereomicroscope.

Isolated embryo sacs were viable and developed normally *in vitro*. Within the first week in culture the embryo sac became enlarged as a result of actively growing endosperm (Fig. 2.4B). The endosperm proceeded to grow into a large

sphere and between the first and second week in culture a torpedo-shaped proembryo became visible (Fig. 2.5A). Viable sections were easily distinguished by the presence of endosperm from sections that were unable to develop (Fig. 2.5B,C). Embryos followed the *in vivo* developmental pattern resulting in embryos with normal structures (Fig. 2.6A,B), which could be germinated by allowing them to remain attached to the endosperm or by rescuing and germinated alone (Fig. 2.6C). The fact that embryo sacs produced embryos and endosperm and cleaved infected FDA (data not shown) indicates that the isolation method was relatively harmless and yielded viable embryo sacs, capable of producing plants (Fig. 2.6D).

Vibratome-isolated embryo sacs were more productive on MS medium containing a relatively high concentration of sucrose and the hormone BAP. More embryos were produced on media containing 15% sucrose than media containing 6% sucrose (Table 2.1). The hormone BAP stimulated a slight increase in endosperm and embryo development at both sugar concentrations. Another obvious difference in the response of Vibratome isolated sections to the media was the growth of the ovary wall. On media with 6% sucrose the ovary wall became very large and callus-like making it difficult to see the embryo sac. As well, this type of growth often twisted the sections resulting in the nucellus being lifted off the medium. As a result endosperm and embryo development was significantly lower with the ovary wall left intact (Table 2.1). When the ovary wall was removed prior to *in vitro* culture on 6% sucrose, however, both endosperm and embryo development increased significantly. Another difference was the extent of endosperm growth on 6% sucrose compared to growth on 15% sucrose. On 6% sucrose the endosperm proliferated into callus, whereas on 15% sucrose the endosperm developed into small dense spherical structures. When embryos were allowed to remain attached to the endosperm on 6% sucrose they would also develop into callus. For this reason embryos were rescued to prevent callus formation. When cultured on 15% sucrose in contact

with endosperm, embryos would not form callus and therefore did not need to be rescued.

Approximately 90% of the embryo sacs produce endosperm within the first week in culture (Table 2.1). The remaining embryo sacs fail to grow, except rarely an embryo sac was observed to produce an embryo without first growing endosperm. From the embryo sacs that produce endosperm 75% produce embryos. To determine if the failure to observe embryos in the remaining 25% was due to the abortion of the zygote, embryo sacs were fixed after 4 days in culture, embedded in paraffin and sectioned. Examination of sections revealed that zygotes had produced globular proembryos. All of the embryo sacs observed had globular-shaped proembryos of various sizes. The failure is likely to have occurred between the globular proembryo and the differentiated embryo. To test this, embryo sacs were fixed after 16 days in culture when embryos were apparent in some embryo sacs. Sectioning of these embryo sacs revealed that some of the proembryos failed to differentiate into embryos. This is not surprising since the components to the regeneration medium (e.g. hormones, osmolality, etc.) had not been optimized to promote embryo differentiation. Modifications of media may increase the number of embryos produced.

DISCUSSION

In developing this technique a primary concern was the viability of the isolated embryo sacs. Embryo sac esterases were able to cleave FDA, which indicated metabolic viability (Heslop-Harrison and Heslop-Harrison, 1970). A more important test of viability, however, was whether the embryo sacs could survive *in vitro* and continue to develop normally. Indeed the embryo sacs developed *in vitro*, with development of the endosperm and embryo mimicking that *in vivo*. Fully-developed embryos were produced on the modified MS medium both with and without the addition of hormones.

The success of Vibratome-isolated embryo sacs was highest on 15% sucrose in the presence of the hormone BAP. High sucrose concentration has

been demonstrated to increase the percentage of zygotic embryogenesis in isolated embryo sacs of maize (Mol et al., 1993). Similarly, BAP has also been shown to have a positive effect on *in vitro* zygotic embryogenesis (Campenot et al., 1992). As well, *in vivo* cytokinin levels are high in fertilized maize ovules up to 8-9 DAP (Lur and Setter, 1993) indicating that cytokinins may be essential for *in vivo* zygotic development.

All embryos examined were zygotic and there was never more than one embryo produced per embryo sac. Somatic embryogenesis would have resulted in the production of more than one embryo per embryo sac. Embryogenesis simulated that *in vivo* with the zygote dividing to form a proembryo and the proembryo dividing and differentiating through transition, stage 1 and stage 2 embryos (Abbe and Stein, 1954). At no stage was a callus intermediate observed. Most of the existing methods for producing transgenic maize involve an intermediate callus stage, yet avoidance of callus is considered important for plant transformations. The somaclonal variation that occurs during callus proliferation (Phillips et al., 1994) is considered undesirable when producing true to type plants (Vasil, 1988). Since regeneration from Vibratome-isolated embryo sacs occurred without callus, transformation of these embryo sacs would be desirable.

The fact that embryos could develop on basic media without addition of hormones suggests that an endogenous source of hormones was present. A completely isolated embryo sac is greatly dependent on its *in vitro* environment for survival. A partially isolated embryo sac however, may be supplied with essential growth stimuli from the remaining maternal tissue (Campenot et al., 1992) In the case of Vibratome-isolated embryo sacs, some nucellus and ovary wall remain to give both structural support and provide nutrients and stimuli for development. Leduc et al. (1995) reported that maize embryo sacs appear to lose their viability during isolation as a result of their exposure to digestive enzymes. Their study also indicated that the structural integrity of the embryo sac is important for long term viability and that adjacent nucellar cells may play

an essential role in stimulating development. For Vibratome-isolated embryo sacs growth stimuli were initially thought to be provided by the ovary wall and nucellus, since the zygote was likely incapable of forming an embryo without external signals. Removal of the ovary wall, however, had no effect on embryo sac development, indicating that the nucellus was largely responsible for initiating development. With the ovary wall intact the lack of growth that occurred on 6% sucrose was caused by twisted growth that removed the nucellus from the medium causing embryo sacs to starve and become dehydrated.

An interesting but rare observation was that embryos would develop from embryo sacs cut by the blade during isolation. Surprisingly these embryos grew in the absence of endosperm. In this situation the central cell was damaged during isolation but the egg apparatus was unharmed. The unharmed zygote then proceeded to develop in the absence of endosperm obtaining its nutrients from the culture medium. This observation supports the idea that the endosperm is not essential for embryogenesis to occur. Leduc et al. (1996) produced plants in the absence of endosperm by co-culture of zygotes with androgenic microspores from barley. As well, somatic embryos form readily without endosperm present. Endosperm may therefore function solely as a nutritive tissue. What then is responsible for influencing the zygote to develop? Are simple nutrients the only requirements or are hormones required? Hormones are included in both the barley androgenic co-culture and in somatic embryogenesis protocols. It is probable that the supportive tissue is providing the zygote with essential signals for development in Vibratome sections. During *in vitro* culture the nucellus progressively degrades around the embryo sac. Perhaps this degradation results in the production and/or release of hormones which influence the development of the associated embryo sac. *In vivo* cytokinin levels drop around 8 DAP in conjunction with a rise in auxin levels (Lur and Setter, 1993). Auxins have been demonstrated to initiate embryogenesis and establish embryo polarity (Liu et al., 1993).

Vibratome-isolated embryo sacs are easily manipulated. The remaining ovary wall and nucellus not only provide a natural environment for embryo sacs to develop but also a means by which embryo sacs can be handled. The sections are thin enough to see cellular detail within the embryo sacs and wide enough to grasp without harming the embryo sacs. The ovary wall and nucellus hold the embryo sacs and prevent them from moving around when cultured *in vitro*. Embryo sacs therefore do not need to be held with suction pipettes or embedded in low-melting-point agarose when microinjected. Since the embryo sacs remain enclosed in nucellus they can be cultured on semi-solid agarose medium without becoming desiccated. Vibratome-isolated embryo sacs are more transparent than those surgically isolated by Campenot et al. (1992). They may however be less transparent than embryo sacs isolated using digestive enzymes (Mol et al., 1995), but do not need to be embedded in agarose to be manipulated.

Vibratome-isolated embryo sacs can be used for several applications. The events of fertilization can easily be studied with the advantage of the embryo sacs being enclosed in their natural state. As well, pre- and post-fertilization barriers may be avoided by *in vitro* fertilization which may lead to interspecific crosses not previously possible. Vibratome-isolated embryo sacs are suitable targets for genetic manipulation. Transgenes may be introduced into embryo sacs directly using any of the direct gene transfer methods that can penetrate through a few cell layers (e.g. microinjection or microprojectile bombardment). Using these methods naked DNA or protein-bound DNA may be directly inserted into the cytoplasm or nuclei of embryo sac cells. DNA-binding proteins with nuclear localization signals (NLS) may help to localize introduced DNA into nuclei (Citovsky et al., 1994). An example would be to directly deliver the T-DNA complex from *Agrobacterium* sp. (Jasper et al., 1994). This complex contains proteins that target the T-DNA to the nucleus (e.g. VirD2 and VirE2) and assist in its integration (Zupan and Zambryski, 1995). Alternatively Vibratome-isolated embryo sacs may be subjected to *Agrobacterium*-mediated transformation.

Embryo sacs could be co-cultivated with *Agrobacterium* similar to immature embryos (Ishida et al., 1996) at any early stage of development starting from the zygote.

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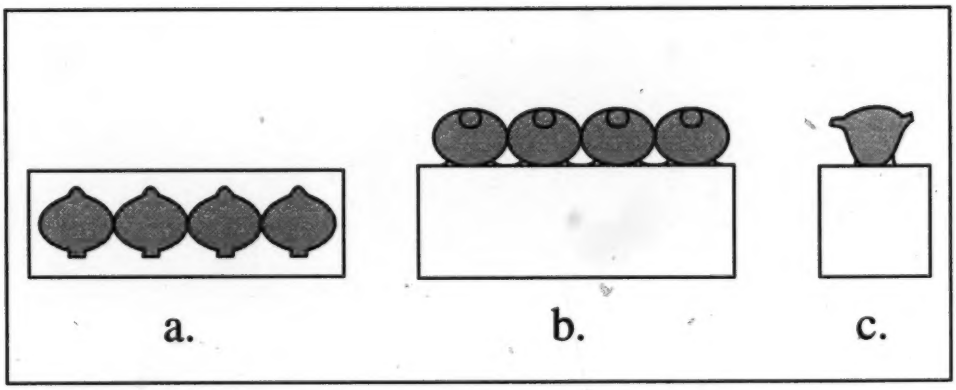

Fig. 2.1 Orientation of ovaries on specimen blocks prior to sectioning.

a) Top view, b) Front view, c) Side view.

Fig. 2.2 Position of ovaries in the Vibratome. The stylar ends of each ovary face the blade. Sectioning is achieved when the vibrating blade advances through the ovaries. Once through, the blade is reversed and the sections collected. Note that sections usually stick to the blade and can be easily removed with forceps.

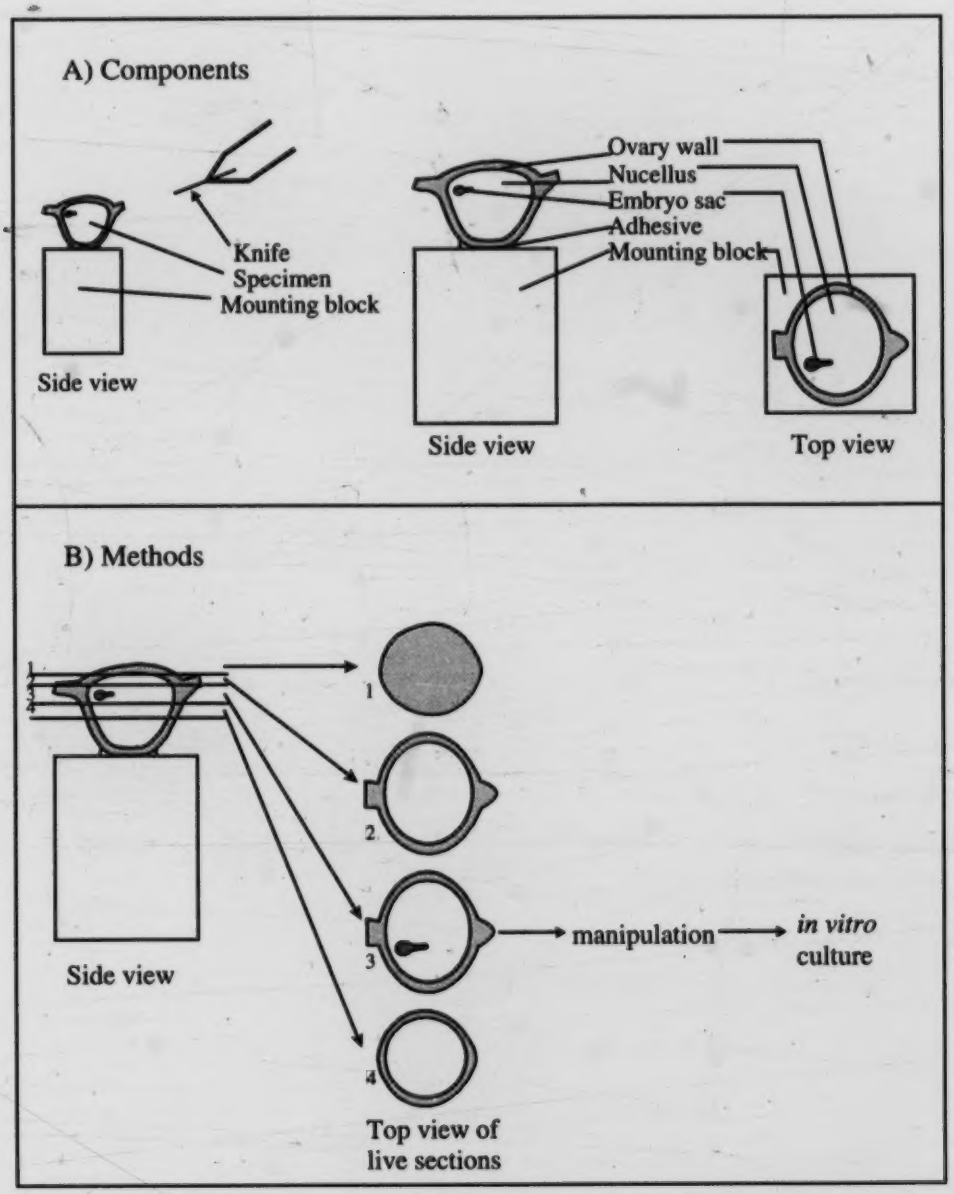

Fig. 2.3 Methodology: A) Ovaries are attached to specimen blocks by an adhesive and placed in the Vibratome. B) Serial sectioning results in the partial isolation of embryo sacs, making them accessible and easily manipulated.

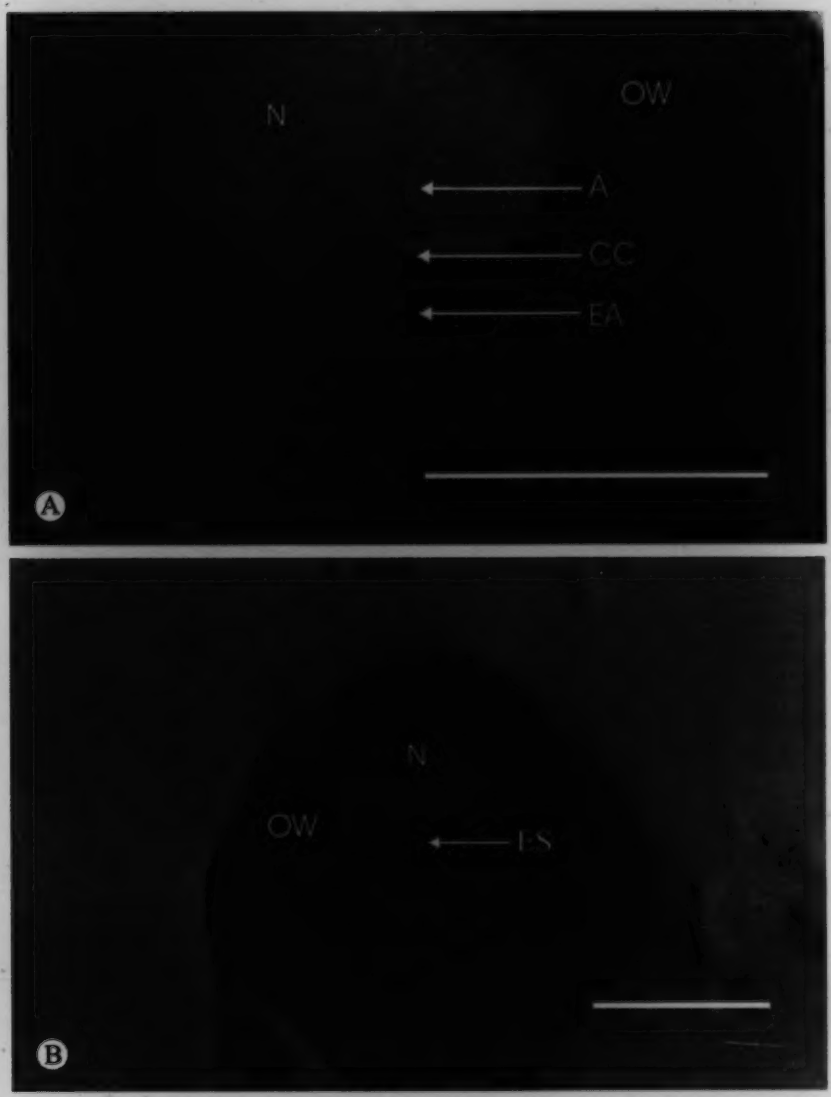

Fig. 2.4 Vibratome-isolated embryo sacs in culture on MS15 medium. A) A freshly cut section containing an intact embryo sac. Bar = 250μm. B) During the first week in culture the rapidly growing endosperm becomes visible. This section contains an embryo sac isolated at 1 DAP and cultured for 4 days on MS15. Bar = 250μm. *Abbreviations:* A, antipodals; CC, central cell; EA, egg apparatus; OW, ovary wall; N, nucellus.

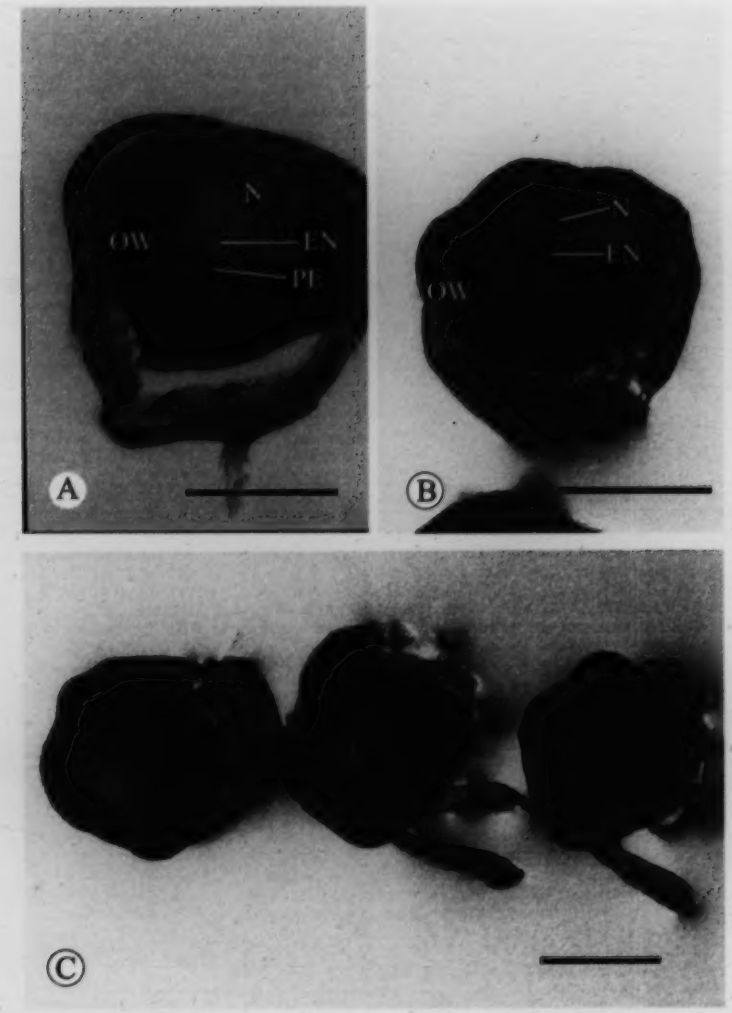

Fig. 2.5 Growth of embryo sacs *in vitro* on MS15. A) During the second week in culture a transition/proembryo becomes visible while the endosperm continues to grow rapidly. B) Endosperm grows into a sphere during the second week in culture. C) Viable embryo sacs can be distinguished from nonviable ones by the presence of endosperm. The section in the middle contains a sphere of growing endosperm while the others do not. *Abbreviations*: EN, endosperm; N, nucellus; OW, ovary wall; PE, proembryo. Bar = 2 mm.

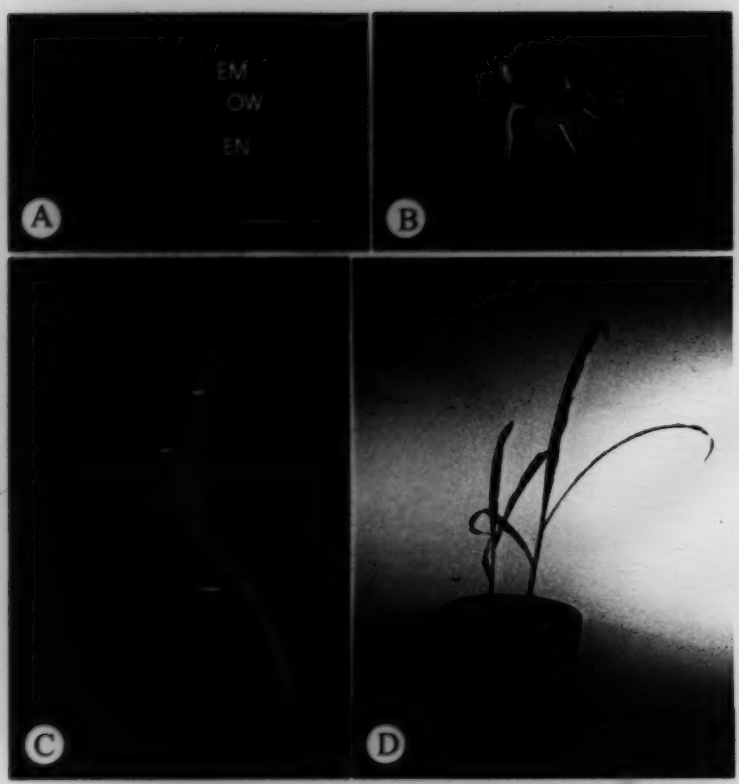

Fig. 2.6 Regeneration of plants from 1 DAP embryo sacs. A) A Stage 1 embryo grown *in vitro* from a vibratome isolated embryo sac. This embryo developed directly from the zygote. Bar = 2 mm. B) A rescued embryo derived from an isolated 1 DAP embryo sac. Bar = 1 mm. C) A rescued embryo that has germinated in the dark. Bar = 5 mm. D) A plant that has developed from an isolated embryo sac. *Abbreviations:* EA, embryo axis; EM, embryo; EN, endosperm; OW, ovary wall; R, root; S, shoot; Sc, scutellum.

Table 2.1 Growth of 1 DAP embryo sacs on various MS media. Values are in percent ± standard error.

Media: % sucrose (6/15), BAP presence (+/-).	Endosperm produced per section in percent.	Embryos produced per section in percent.
MS6-	10.0±2.05	8.1±2.22
MS6+	15.3±0.71	12.1±2.31
MS6-*	79.3±0.74	55.3±4.67
MS6+*	89.9±2.10	69.5±1.76
MS15-	74.2±2.88	60.7±11.35
MS15+	81.4±6.44	75.0±3.61

* sections containing embryo sacs surrounded by nucellus with the ovary wall removed.
Statistically significance at $P<0.05$.

CHAPTER 3

Effects of Sucrose Concentration and 6-Benzylaminopurine on *In Vitro* Development of Maize Embryo Sacs and Embryos

INTRODUCTION

In vitro fertilization and the isolation of zygotes from maize have created a demand for reliable *in vitro* regeneration (Faure et al., 1993; Kranz and Lorz, 1993). Fusion products must be stimulated to undergo zygotic embryogenesis and subsequent germination to produce plants. *In vitro* culture protocols should promote normal development with little or no callus production, and once embryos have germinated they must be capable of maturing to sexually competent plants. Such a system will allow the study of embryogenesis and make possible the regeneration of genetically transformed endosperm, zygotes, and gamete fusion products (Campenot et al., 1992; Leduc et al., 1996).

Embryogenesis has been studied extensively in maize (Van Lammeren, 1987). The *in vivo* events of cell division and differentiation from the zygote to a mature embryo have been well documented (Abbe and Stein, 1954). *In vitro* culture of embryo sacs and immature embryos, however, is more difficult and is the topic of numerous studies (Mol et al., 1995). Dahmen and Mock (1972) were able to isolate and culture embryos 13-16 days after pollination (DAP). Van Lammeren (1987) had limited success at culturing embryos younger than 10 DAP. Recent success however, has been achieved for isolated 1 DAP embryo sacs and zygotes (Campenot et al., 1992; Leduc et al., 1995; Leduc et al., 1996). Development was promoted by androgenic feeder (nurse) cultures or by media supplemented with growth hormones (Kranz et al., 1991; Petersen et al., 1992). The disadvantage of using nurse cultures is that there is far less control over nutrients and growth regulators influencing embryonic growth. Recent evidence supports the idea that nucellar cells enhance formation of embryos (Leduc et al., 1995). Removal of these cells during isolation adversely affects the differentiation and development of zygotes to embryos. By studying isolated embryo sacs growing in the absence of nucellus, one may gain knowledge of the

nutritional and hormonal factors required to initiate differentiation and development.

The current study was designed to investigate the effects of different concentrations of sucrose and a cytokinin, on immature embryos and on maize embryo sacs containing proembryos. Two different concentrations of sucrose and various concentrations of the cytokinin, 6-benzylaminopurine, were added to a modified Murashige and Skoog medium. Embryo sacs and embryos were excised and cultured on various media. Treatments were set up to initiate growth of proembryos into differentiated embryos, and growth of immature embryos into normal, healthy plantlets. Five DAP embryo sacs were selected because of their relative ease of isolation. At this stage the whole embryo sac can be surgically removed from the nucellus. The embryo sac is filled with endosperm tissue except for a proembryo in the micropylar end. The embryo sacs can be placed in culture and manipulated to promote development. Similarly immature embryos can be isolated with ease and placed in culture. Manipulation of embryos by changing culture conditions may provide an insight into how they behave *in vivo.* As well, the activity of certain genes involved in embryo maturation and germination can be studied.

MATERIALS AND METHODS

Plant Material

Zea mays (L.) plants were grown in a pollen-free environment with their tassels removed well before anthesis. At maturity, cobs were hand pollinated. Silks were trimmed to approximately 6 cm beyond the tips of the husks, and thoroughly covered with pollen. The cobs were then bagged to prevent further pollination. Cobs were harvested at 5 and 10-13 DAP.

Isolation of Embryo Sacs and Immature Embryos

After removing the husks and silks, the cobs were surface sterilized for 10 minutes in 70% ethanol, followed by 3 rinses of sterilized distilled water. Using

sterile technique and working in a laminar air flow hood the embryo sacs (5 DAP) or embryos (10-13 DAP) were surgically removed, and plated on a modified Murashige and Skoog (MS) medium with 0.4mg/liter L-asparagine and agar (7 g/liter) at pH 5.8. Embryo sacs excised from ovules at 5 DAP were placed on MS3 (3% sucrose), MS6 (6% sucrose), MS3 + 0.1BAP (0.1 mg/l BAP) and MS6 + 0.1BAP. The explants were maintained at 23°C in the dark and observed for signs of embryo and endosperm growth. Embryos excised from ovules 10-13 DAP were placed scutellum down on MS media. The media were MS3, MS6, MS3 plus 3% mannitol, MS6 plus 3% mannitol, and MS3 containing various concentrations of BAP (1.0, 0.1 or 0.01 mg/l). As well, embryos were exposed to MS15 for 24hours prior to culture on MS3 and MS6. All embryos were cultured in the dark at 23°C, and monitored for signs of callus, root and shoot growth. Triplicates of thirty embryo sacs and embryos were used to calculate mean and standard error for each treatment.

TTC assay for viability

Isolated embryo sacs and embryos were incubated in TTC (0.6% w/v 2,3,5-triphenyltetrazolium chloride in phosphate buffer at pH 6.8) overnight (Towill and Mazur, 1975). Observations were made with a Zeiss stereomicroscope (Stemi SV11). Red color indicated cell viability, whereas the absence of color indicated cell death.

RESULTS

Embryo sacs at 5 DAP were easily isolated and placed in culture on MS medium (Fig. 3.1A). These embryo sacs were apparently viable since they were red in the TTC assay (Fig. 3.1B). After one week in culture embryo sacs either split open due to growth of white, fluffy endosperm derived callus or remain unchanged (Fig. 3.2). More embryo sacs produced endosperm callus on MS medium supplemented with 6% sucrose than with 3% sucrose (Table 3.1). As well, the callus grown on media without BAP (MS6) was twice the diameter of callus grown on media with BAP (MS3 or MS6), which was more brown in color.

After an extended culture period the callus increased in size and root growth became apparent in each of the treatments (Table 3.1).

Embryos isolated 10-13 DAP were observed daily. After one week in culture differences were observed between embryos grown on MS6 and MS3 (Fig. 3.3). The embryos cultured on MS6 appeared healthy with a well developed scutellum and embryo axis. Embryos cultured on MS3, however, had virtually no scutellum, but had an apparently healthy embryo axis. Embryos cultured on MS6 supplemented with BAP exhibited abnormal shoot growth (Fig. 3.3D). These embryos had reduced scutella, extensive trichome development and extensive leaf initiation (shoot proliferation). As well, they were smaller than embryos cultured without BAP. After three weeks in culture many of the embryos germinated. Germination consisted of the production of a root and a shoot. Shoot growth was obvious in all the treatments, with normal shoot growth in the treatments without BAP, and abnormal shoot growth with extensive leaf growth in treatments with BAP (Fig. 3.3D). Poor root growth occurred in embryos cultured on MS6, MS3 and MS3 + 1.0BAP. When roots failed to grow the basal end of the embryos became swollen (Fig. 3.4). These swollen root apices were more numerous on MS6 than on MS3 media. Embryos cultured on media with 3% mannitol also produced enlarged root apices. MS3 plus 3% mannitol was comparable to MS6, but MS6 plus 3% mannitol inhibited root growth to the greatest extent (Table 3.2). A 24-hour pretreatment on MS15 prior to *in vitro* culture on MS3 or MS6 resulted in rapid germination. Both shoot and root growth were apparent; no swollen root apices were observed (Fig. 3.4B).

DISCUSSION

Culture of 5 DAP Embryo Sacs

In vitro culture of embryo sacs containing proembryos (5 DAP) resulted in extensive endosperm callus growth, but very little proembryo differentiation. Endosperm callus formed within one week of culture on all the different media (Fig. 3.2). Since no nucellus was present the observed growth was due to

endosperm or proembryo or both. The callus originated from a central location in the embryo sacs, and was likely a product of the endosperm, not from the proembryo. Growth of endosperm in partially isolated embryo sacs *in vitro* is common (Campenot et. al., 1992; Leduc et al., 1995). BAP had no obvious effect on endosperm initiation, but did effect endosperm growth. Endosperm cultures are usually initiated on hormone-free media (Shannon, 1994). The sugar concentration appears to be more important for initiating endosperm growth. Better results obtained with higher concentration (MS6) may be due to greater availability of sugar or its higher osmolarity. This was an unexpected result since Shannon (1994) used 3% sucrose to initiate endosperm cultures.

The experiments were designed to stimulate normal zygotic embryogenesis of the proembryos. Unfortunately, only a few roots were observed with no apparent differences between treatments. It is likely that only root organogenesis occurred instead of complete embryogenesis. The roots which developed were located near the periphery of the calli toward the micropylar end of the embryo sac and likely originated from the proembryo. BAP was selected to initiate development since it is useful for initiating development of isolated zygotes and partially isolated embryo sacs containing zygotes (Campenot et al., 1992; Leduc et al., 1995). The exact origin of the roots was not determined. The roots could have formed from callus. The disadvantage of organs forming from callus is that callus tissue is not homogeneous. Somaclonal variation in callus can produce inferior organs. Since no callus was observed besides the endosperm it is likely that the roots formed directly without prior callus.

All the media used promoted growth of callus from endosperm. It is possible that this callus suppressed the growth of the proembryo. Van Lammeren (1987) reported root growth and suppression of shoot growth from proembryos cultured *in vitro* in contact with endosperm, and both root and shoot growth in the absence of endosperm. The success of Campenot et al. (1992) at culturing proembryos was likely due to their isolation procedure. Their explants

were surrounded by a few cell layers of nucellus, which may have synthesized substances promoting differentiation of proembryos and minimizing the suppressive influence of the endosperm. Since separation of proembryos from endosperm is difficult, an *in vitro* culture system that would promote differentiation of proembryos and suppression of endosperm callus would be advantageous.

Currently, maize somatic embryos are derived from immature embryos (Lu et al., 1983; McCain and Hodges 1986; Emons and Kieft, 1991). The synthetic hormone 2,4-dichlorophenoxyacetic acid (2,4-D) is used to stimulate embryo differentiation at low sucrose concentrations. Once somatic embryos differentiate, they are placed on a medium lacking hormones but with a high sucrose concentration to promote maturation. After a period of maturation the embryos are transferred to another medium lacking hormones, but with a low concentration of sucrose. These procedures mimic the events that occur during zygotic embryogenesis *in vivo*. The present study provides evidence that the presence of BAP does not stimulate development of embryos from proembryos. Perhaps an auxin would be more suitable. Campenot et al. (1992) and Van Lammeren (1987) cultured zygotic proembryos on 2,4-D and failed to get differentiated embryos. Both groups, however, incubated the embryo sacs on auxin for extended culture periods. A better approach might have been to incubate embryo sacs on auxin-enriched media for a shorter period as done for somatic embryos. Exposure of immature embryos to exogenous auxins is known to promote callus formation (Welter et al., 1995). Removal of embryos from media containing hormones after embryo differentiation is, therefore important for preventing callus formation.

To initiate normal zygotic embryogenesis in maize a number of things should be considered. At five DAP the embryo sac contains a globular, undifferentiated proembryo. Between five and eight DAP the proembryo differentiates a root apex, a shoot apex and a scutellum. An *in vitro* culture system must promote the differentiation of proembryos to embryos. Mature

embryos should then be placed on an appropriate medium to stimulate germination. The present results indicate that BAP is not suitable for stimulating differentiation of proembryos into embryos. As for somatic embryos, a short incubation period on an auxin (i.e.2,4-D) should be tested for stimulating differentiation to embryos.

Culture of 10-13 DAP Embryos

Initially, the immature embryos appeared to develop on MS6 medium. The embryo axis as well as the scutellum enlarged and became less transparent prior to germination. Precocious germination, however, was initially more frequent among embryos on MS3. The scutellum degenerated on these embryos while the embryo axis enlarged. Older zygotic embryos as well as somatic embryos are known to remain in a developmental mode when cultured on a high osmoticum (Xu et al., 1990), but germinate precociously on a low osmoticum (Kermode and Bewley, 1989). MS6 apparently had too low a sucrose concentration to maintain embryos in the developmental mode. Even MS6 plus 3% mannitol could not entirely prevent germination. Isolated immature embryos of maize will however remain in a developmental mode when cultured on media containing 15% sucrose or higher (Delvallee et al., 1989).

After one week in culture germination was evident in all treatments. Shoot growth was common, whereas root growth was variable. Shoot growth was similar in both the MS3 and MS6 treatments, but root growth was greater on MS3 media. Higher sucrose concentration increased the osmolarity of the medium and may have caused water stress that decreased the endogenous auxin levels in the embryos (Ashburner et al., 1993). Lower endogenous auxin levels may have prevented root formation. Embryo enlargement in the region of the root apex may have increased the surface area for absorption which would serve to support the developing shoot. The few embryos that did form roots may have been more resistant to water stress and therefore produced sufficient auxin. Since root growth was also low for embryos plated on MS3, the lack of a maturation period may be the cause of poor root formation. Perhaps the root

apex was not fully developed or was unable to respond to conditions favoring germination. Embryos at this stage of development would not normally germinate, but do so when isolated and placed on media with nutrients and appropriate water potential.

Campenot et al. (1992) produced embryos *in vitro* from 1 DAP partially isolated embryo sacs on BAP enriched media. They cultured these embryos continuously on media supplemented with BAP. The present study was designed to investigate the effects of BAP on differentiated but immature embryos. After one week in culture the embryos plated on MS3 medium with BAP showed abnormal shoot growth. High levels of certain cytokinins are known to inhibit somatic embryogenesis in celery, peanut and certain grasses and therefore may have a detrimental effect on zygotic embryogenesis (Van Staden et al., 1992; Eapen and George, 1993). The embryos on MS3 plus BAP were much smaller than those plated on MS6, and produced many leaves and trichomes. BAP stimulated premature initiation of leaves, with more leaves being produced as the concentration of BAP increased. Cytokinins are known to stimulate shoot proliferation (Zhong et al., 1992).

After three weeks in culture, roots had grown from the embryos. Root growth was greatest at lower concentrations of BAP, and was likely stimulated by the production of leaf hormones. Young leaves are known to produce high levels of auxins. Higher concentrations of BAP may have maintained a high cytokinin to auxin ratio, thus preventing significant root formation. The number of leaves was much lower for embryos cultured on media without BAP; this may have attributed to poor rooting in these treatments. By simply looking at the number of embryos producing roots and shoots, one might consider the MS3 medium with a low concentration of BAP to be the best medium for regeneration of immature zygotic embryos. Without growing these embryos to maturity, however, the long term effects of shoot proliferation are unknown. Perhaps these embryos will produce shorter, leafier plants with premature flowering.

A 24 hour incubation of immature embryos on MS15 followed by *in vitro* culture on MS3 or MS6 resulted in precocious germination. However, germination consisted of both root and shoot growth without any basal swelling of embryos. Premature drying is known to initiate precocious germination of immature embryos (Oishi and Bewley, 1992), but not at such an early stage of development when embryos are desiccation intolerant. Moderate dehydration on MS15 may have activated genes involved in germination which are normally expressed during imbibition after maturation drying (Bewley and Marcus, 1990). The effect is greatest in the root apex, since shoot apex growth occurs without prior dehydration. This means that after dehydration the root apex is more responsive to growth factors which may be a result of the developmental switch from maturation to germination. This phenomenon should be studied in more detail to determine if indeed the embryos have upregulated expression of germination genes after dehydration, and why the effect is most noticeable in the root apex.

Differentiated immature zygotic embryos of maize appear to react similarly to somatic embryos in culture. Maturation is achieved on a medium with a high sucrose concentration, and germination is initiated on a medium with a low sucrose concentration. The cytokinin BAP causes abnormal shoot growth by stimulating shoot proliferation. After an extended period of culture, however, the embryos on BAP produce more roots than those not on BAP. The long term effects of shoot proliferation at such an early stage of development should be studied. Abnormal shoot growth might be avoided by improving the maturation and post-maturation culture conditions for immature embryos. Alternatively, premature dehydration may be used to increase germination. These results along with those from culturing embryo sacs containing proembryos take us one step closer to understanding normal zygotic embryogenesis *in vitro*.

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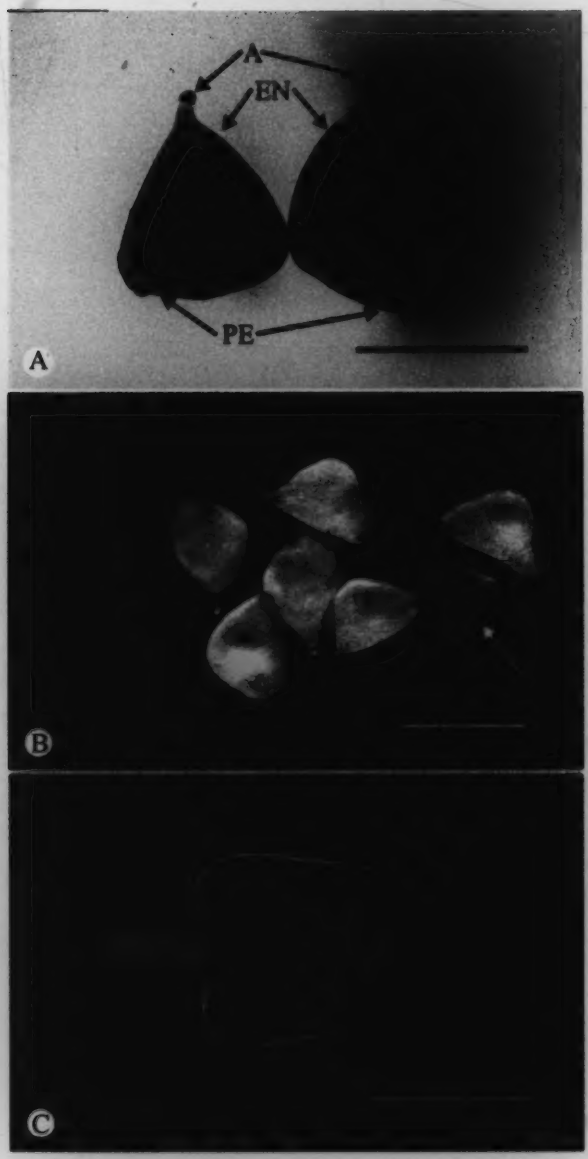

Fig. 3.1 Freshly isolated embryo sacs and embryos. A) Embryo sacs isolated at 5 DAP containing antipodals (A), endosperm (EN) and proembryo (PE). Bar = 1 mm. B) Five DAP embryo sacs. One embryo sac was incubated in TTC for 2 hours (arrow). Bar = 1.5 mm. C) Immature embryo isolated at 10 DAP. Note the coleoptile (arrow), root apex (RA), scutellum (Sc), and the shoot apex (*). Bar = 750 μm.

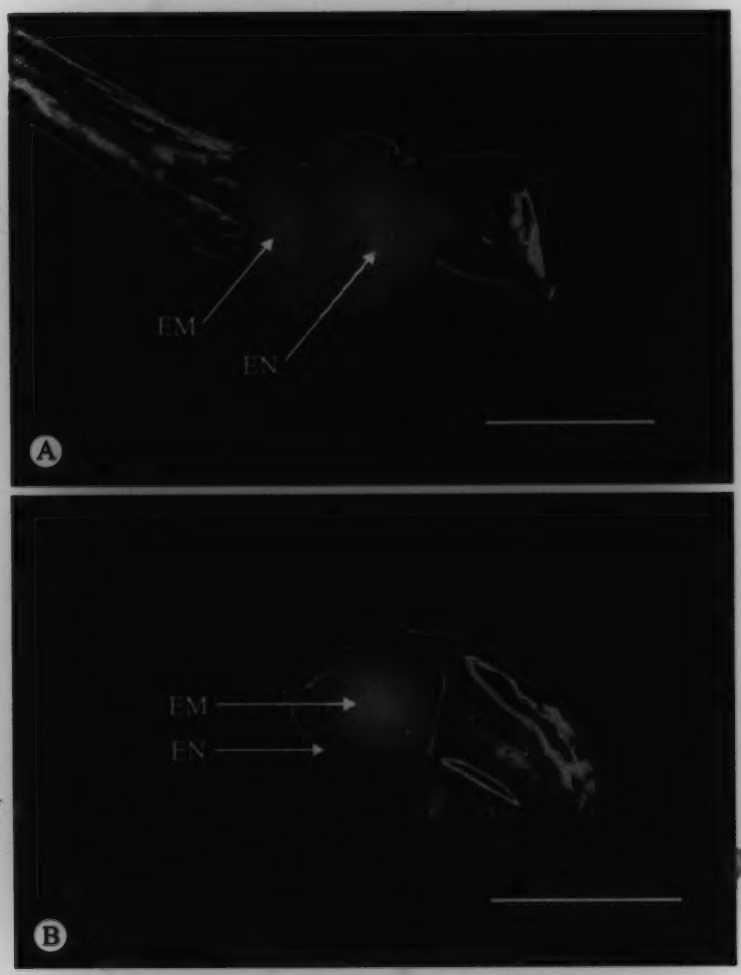

Fig. 3.2 Maize embryo sacs in culture for 11 days on MS6 after isolation at 5 DAP. A) Endosperm (EN) and proembryos (EM) each produce dense callus. Bar = 1.5 mm. B) Occasionally the endosperm (EN) fails to produce callus but the embryo grows into a apparently unorganized structure (EM). Bar = 2 mm.

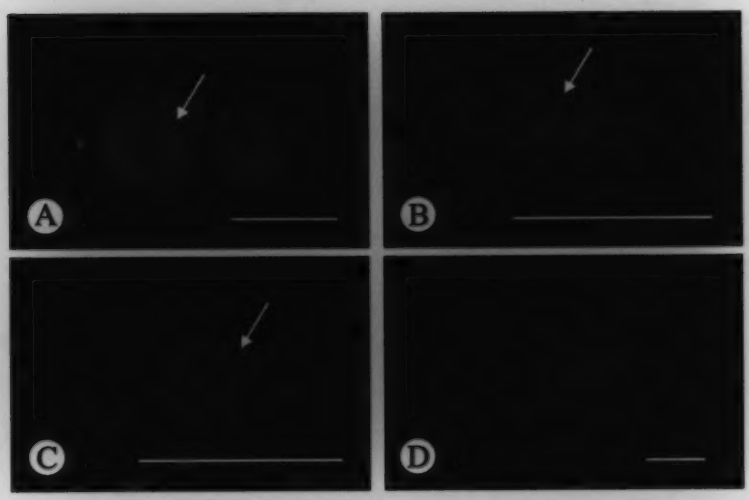

Fig. 3.3 Growth of 10 DAP embryos on MS containing either 3 or 6% sucrose with or without BAP. A) Ten DAP embryos cultured for 2 days on MS6. Note the enlarged scutella (arrow). B) Ten DAP embryo cultured for 2 days on MS3. Note the reduced scutellum (arrow). C) Ten DAP embryo cultured for 3 days on MS6+BAP. Note the shoot proliferation and trichome development (arrow). D) Ten DAP embryo cultured on MS6+BAP for 2 weeks. Note shoot proliferation (arrow). Bars = 2 mm.

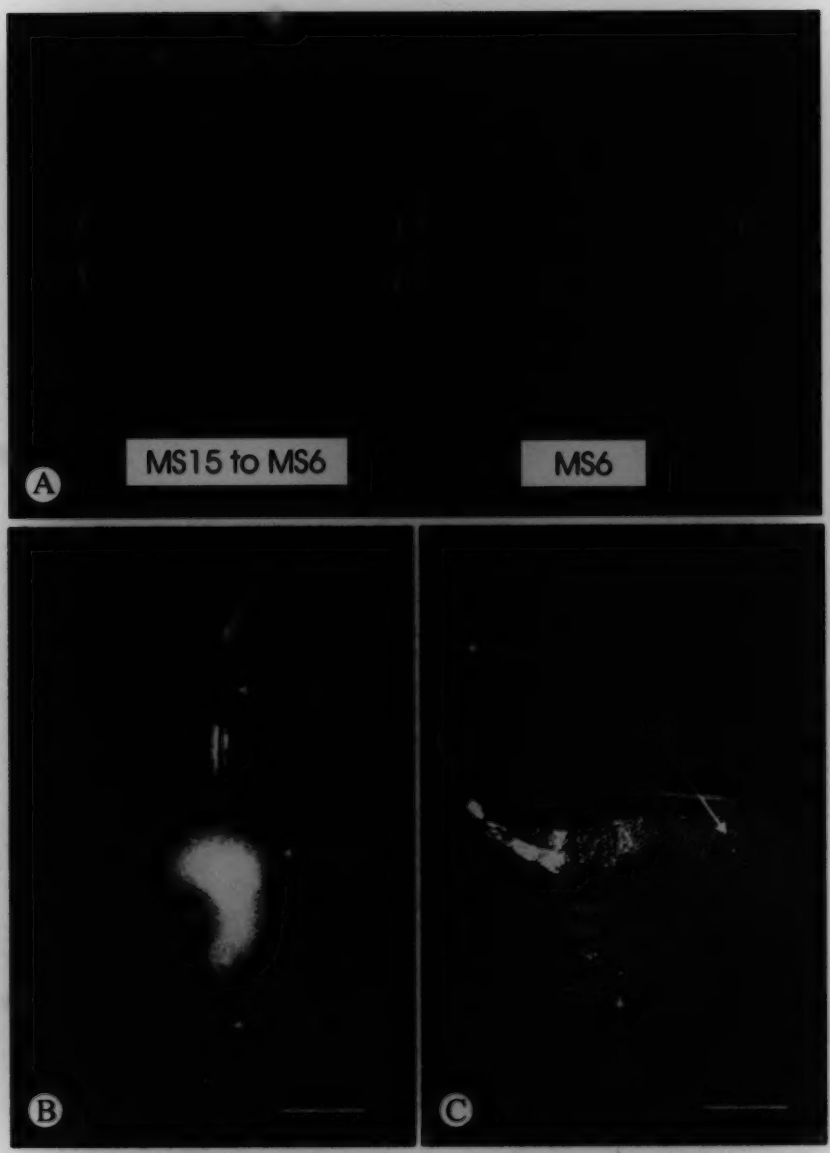

Fig. 3.4 Dehydration promotes germination of maize immature embryos. A) Twelve DAP embryos cultured on MS6. The dish on the left contains embryos cultured on MS15 for 24 hours prior to culture on MS6 for 4 days. The dish on the right contains embryos cultured for 5 days on MS6. Note the enlarged scutella and root apices and the lack of roots (arrow). B) A germinating embryo treated as the dish on the left above. C) Embryo with enlarged root apex and scutellum treated as the dish on the right above. Bars = 2 mm. *Abbreviations:* ER, enlarged root apex; R, root; S, shoot; Sc, scutellum.

Table 3.1 Growth of 5 DAP embryo sacs on various MS media.

Growth media	Growth of endosperm	Growth from proembryo[1]
MS3	28.8±1.91*	8.2±1.08
MS3+BAP	34.4±8.69	11.9±2.40
MS6	81.6±9.23	15.0±4.17
MS6+BAP	76.3±13.15	10.4±0.37

[1]Growth was indicated by embryogenesis, organogenesis, or by callus formation.
*Values in percent ± standard error.

Table 3.2 Growth of maize immature embryos after 7 days on various MS media.

Growth media	Germination[1]	Scutellar growth[2]	Shoot growth
MS3	61.8±9.04	Degenerate	Normal
MS6	49.1±11.08	Developed	Normal
MS15	6.47±3.02	Developed	Normal
MS3+3	27.0±3.87	Developed	Normal
MS6+3	15.7±3.67	Developed	Normal
MS3+BAP(.01)	55.1±10.83	Degenerate	Shoot proliferation
MS3+BAP(0.1)	55.6±10.02	Degenerate	Shoot proliferation
MS3+BAP(1.0)	23.5±3.44	Degenerate	Shoot proliferation
MS15 to MS3	92.0±3.99	Degenerate	Normal

[1]Germination was scored as both root and shoot growth.
[2]See text for description of growth.

CHAPTER 4

Cryopreservation of Maize Immature Embryos

INTRODUCTION

Cryopreservation of plant embryos is of great practical interest (Bajaj, 1985) because of their use as tools for experimental biology and importance for mass propagation (Engelmann and Dereuddre, 1988). For incompatible crosses where embryos would normally abort it may be possible to isolate immature embryos, prior to abortion, and grow them in culture. Embryos of recalcitrant seeds can be isolated and preserved indefinitely in liquid nitrogen (Bouchaud et al., 1991). For plants that produce short-lived seeds or very large seeds isolated embryos can be cryopreserved, increasing the length of storage time and reducing storage area. Attempts have been made to cryopreserve zygotic, somatic, nucellar, pollen-derived embryos, and other totipotent tissues from important crop species and from rare and endangered wild species (Iriondo et al., 1992; Mycock et al., 1995). Germplasm conservation is an active field which has both environmental and economic value (Grout, 1990; Stanwood, 1985).

Isolation of immature embryos is becoming increasingly important creating a demand for suitable cryopreservation protocols. Maize immature embryos (Delvallee et al., 1989) as well as immature embryos from other grasses have been cryopreserved (Gnanapragasam and Vasil, 1992). Moisture content is thought to be the major factor limiting survival during freezing (Grout et al., 1983). To avoid or reduce freezing injury various freeze-hardening procedures have been developed. Freeze-hardening may be achieved by preculturing embryos with various cryoprotectants, or by preculturing embryos with various osmotica to promote dehydration. Maturation of embryos in detached cobs has also resulted in some degree of hardiness to freezing (Delvallee et al., 1989). As well, preculture on media containing abscisic acid (ABA) has been demonstrated to increase the resistance of tissues to dehydration and improve their survival after freezing (Reed, 1993; Zeevaart and Creelman, 1988). All of these freeze-

hardening treatments prepare embryos for the dehydration caused by ice formation.

Plant embryos have survived cooling to -196°C without ice formation by vitrification (Uragami et al., 1989). This process is considered less damaging than ice formation during constant rate cooling, but requires use of highly concentrated solutions. Fortunately the same pretreatments used for freeze-hardening also enable embryos to survive dehydration during incubation with concentrated vitrification solutions. The present study explores the use of constant rate cooling and vitrification for cryopreservation of maize immature embryos. Fresh and freeze-hardened embryos were used in both methods.

Constant Rate Cooling

Constant rate cooling is the process of slow cooling to some intermediate subzero temperature (eg. -40°C) followed by rapid cooling by direct immersion in LN. During the first step ice forms, thus concentrating solutes in the unfrozen fraction and dehydrating cells (Meryman and Williams, 1985). In the second step rapid cooling is thought to promote vitrification of the unfrozen fraction containing the cells (Rall, 1981). During slow cooling the ability of cells to equilibrate with the unfrozen fraction depends on the osmotic permeability of particular cells (Mazur, 1970). Cells with low osmotic permeability will freeze internally if the cooling rate is too high. If the cooling rate is too low, however, the cells may suffer damage due to solution effects (ie. the concentrated unfrozen fraction). Cells with higher osmotic permeability can be cooled at higher rates without intracellular ice forming. Choosing the appropriate cooling rate for a particular cell type is often difficult and is one disadvantage of constant rate cooling. The complexity of freezing many cell types together is a limiting factor of this method since each cell type in a tissue would likely have a different optimal cooling rate.

Vitrification

Vitrification of an aqueous solution is when the solution is cooled below zero and a phase transition to an apparent solid is accomplished without ice formation. As the solution cools its concentration remains unchanged as its viscosity increases. At some point its viscosity becomes so high that molecular motion nearly stops. The result is an amorphous state referred to as a glass (Fahy, 1988). This glass transition occurs over a limited temperature range and is a second order phase transition.

Contrary to freezing, vitrification is not considered damaging to living systems (Fahy 1988). The highly concentrated solutions required to achieve vitrification, however, can be damaging. If the biological system is able to survive equilibration with the concentrated solution then it will likely survive the cooling process while forming a glass. The solution concentration does not change during the vitrification process, and can be easily manipulated prior to cooling. Loading and unloading of cryoprotectants may take a long time, but vitrification does not require a programmable freezer and the damage associated with ice formation is avoided.

The present study explores the use of cryopreservation to store maize immature embryos. Embryos were isolated from cobs at 10-14 DAP and subjected to both vitrification and constant rate cooling. The desiccation sensitive nature of the embryos required them to be freeze-hardened. Hardening involved the use of osmotica and ABA to promote dehydration tolerance. The hardened embryos were successfully cryopreserved and regenerated to produce normal, healthy plants. This is the first report that utilizes vitrification to store maize immature embryos.

MATERIAL AND METHODS

Plant Material

Plants of *Zea mays* (L.) cultivar CV129 were grown in a pollen-free environment by removal of tassels before anthesis. Cobs were hand-pollinated

at maturity by trimming silks to 6 cm and thoroughly covering with pollen. The cobs were then bagged to prevent further pollination. Cobs were harvested 10-14 days after pollination (DAP) prior to freezing and vitrification experiments.

Isolation of Immature Embryos

After removing the husks and silks, the cobs were surface sterilized for 10 min. in 70% ethanol, followed by three rinses of sterilized distilled water. Using sterile technique and working in a laminar flow hood the embryos were surgically removed.

Freeze-hardening on Osmoticum

Embryos were freeze-hardened by dehydrating them on various osmotica for 0-5 days. For constant rate cooling the osmotica were composed of basic MS medium with 0.7% agar containing either 15% or 30% sucrose. Otherwise embryos were precultured on MS medium containing 6% sucrose and 5% DMSO. Embryos were also precultured on MS containing 15% sucrose and 50μM abscisic acid (ABA) for vitrification experiments. Controls for all experiments involved culturing embryos on MS medium containing 6% sucrose.

Freeze-hardening by Maturation

Detached cobs were wrapped in aluminum foil and placed in a refrigerator at 4°C for 0-5 days.

Freeze-hardening by Maturation Followed by Dehydration on Osmoticum

After 1-3 days of maturation on the cob at 4°C, embryos were isolated and placed on MS15 medium to promote dehydration.

Freezing and Thawing Procedures

Prior to freezing embryos were incubated in MS medium containing 15% sucrose supplemented with 10% DMSO for 15 min. at 23°C in cryotubes (Nunc,

1.8 ml). Freezing was either rapid by direct immersion in LN or slow (1°C/min.) to -40°C followed by immersion in LN. For all experiments, ice was seeded at -10°C and thawing was achieved by direct transfer of tubes to a circulating water bath at 25°C. Chill sensitivity was determined by cooling embryos to -5°C for 30 minutes without ice formation, followed by warming to room temperature. The effect of ice formation, without deep freezing was determined by slow cooling to -40°C (as above) followed by rapid thawing in a water bath to 25°C. Experiments were performed on freshly isolated and freeze-hardened embryos. Triplicates of thirty embryos were used for each treatment to calculate mean and standard error.

Optimizing Vitrification Solutions

Three commonly used vitrification solutions were selected from the literature and evaluated for their ability to vitrify (Table 4.1). As well, their toxicity to maize immature embryos was determined by incubating the embryos in full strength solutions for various lengths of time with and without phosphate-buffered saline (PBS). Freshly isolated and freeze-hardened embryos were used, and their viability determined by the triphenyltetrazolium chloride (TTC) assay and by *in vitro* culture on MS6. Freeze-hardening involved incubation of embryos at 27°C in the dark on MS15, MS15+50µM ABA and cob maturation (as previously described).

Loading Cryoprotectants & Cooling

Loading of the vitrification solutions was done by incubating the embryos in full strength solutions for 15 min. at 25°C. One ml of the chosen vitrification solution was added to each cryotube (Nunc, 1.8 ml). Embryos were added to the solutions, incubated and the solutions refreshed prior to cooling. During loading the tubes were gently shaken to encourage the embryos to sink. Tube caps were securely tightened prior to placing tubes in aluminum holders and plunging rapidly into LN.

Warming & Unloading Cryoprotectants

Rapid warming was achieved by plunging the samples into a 37 or 45°C circulating water bath. The tubes were held in the water bath for 1 min. before the immediate removal of the cryoprotectants. After warming the embryos were either placed directly on MS medium containing 6% sucrose in the dark or first diluted (washed) with sterile distilled H_2O prior to culturing. Alternatively, one ml of unloading solution was then added to each tube. The unloading solution consisted of 1.2M sucrose with PBS (1X) at pH 5.8. After 20 min. of unloading the tubes were swirled and emptied into a sterile petri dish. The embryos were then placed directly into TTC or on to MS6 with their embryo axes upward. Viability was assessed by growth *in vitro* and by TTC reduction after overnight incubation in the reagent followed by microscopic observation.

Viability Assays

The effect of incubating embryos in the vitrification solutions for various lengths of time was determined by *in vitro* culture and the TTC test. The same procedures were used for determining survival after cooling to -196°C. Embryos were placed in TTC overnight and then observed through a stereomicroscope. A red color indicated presence of viable cells and survival whereas the absence of red indicated lack of viability (Towill and Mazur, 1975). The long term effects were determined by *in vitro* culture on MS6 medium in the dark at 27°C. Embryos were monitored for signs of root, shoot or scutellar growth. Once the embryos germinated the petri dishes were placed in the light until a significant amount of root growth had developed and the leaves were green. The seedlings were then washed free of agar, planted in soil and placed in a growth chamber. Seedlings were covered initially to maintain high humidity. Once they were established the covers were removed and the plants were allowed to grow to maturity.

Vitrification Procedure

The solutions were cooled in glass tubes (Kimble, 6 X 50mm) or Nunc cryotubes. Rapid cooling was done by direct immersion or slowly lowering the tubes into LN. The solutions were considered vitrified when cooled to -196°C without visible ice formation. The presence of ice was indicated by the loss of solution transparency, while vitrified solutions remained transparent. Vitrification was performed on solutions without embryos prior to attempting the procedure on solutions containing embryos. The samples were stored in LN for at least 30 min. after cooling.

RESULTS

Constant Rate Cooling

At 10-14 DAP embryos were 1-2 mm long and could be isolated without a microscope (Fig. 3.1C). Isolated embryos were grown *in vitro* on MS6 medium until they could be transferred to soil; all such embryos grew to maturity.

Freshly isolated embryos could not be successfully cryopreserved (Fig. 4.1). The use of DMSO in combination with rapid or slow cooling rates did not enable these embryos to survive constant rate cooling.

Freeze-hardening by preculture on various osmotica caused desiccation of maize embryos with little damage (Table 4.2). After preculture for 5 days on MS15 embryos developed with no abnormalities when transferred to MS6. Scutellum callusing was observed after preculturing on MS30, but the embryo axis appeared to develop normally. Abnormal growth after preculture on MS6+5% DMSO consisted of callus and stunted growth suggesting that maize embryos are slightly sensitive to prolonged exposure to DMSO.

Freeze-hardening enabled maize immature embryos to survive freezing (Table 4.3). Embryos cooled to -5°C for 30 min. and then rewarmed to 25°C showed negligible chilling injury. Only 80% of the chilled embryos produced primary roots, suggesting differential sensitivity of the root and shoot apices to temperature stress. Embryos cooled to -10°C, nucleated, and then cooled to

-40°C had significantly lower survival rates (30%) upon rewarming to 25°C. Embryos immersed in LN after slow cooling to -40°C were damaged only slightly more than those frozen only to -40°C. Embryos which were directly transferred to LN from 23°C, however, produced no shoots and only 20% had viable roots after thawing.

Freeze-hardening is achieved only after 5 days of pretreatment (Table 4.4). Five days of maturation of embryos in detached cobs resulted in callusing of 13% of the embryos after freezing. After 5 days preculture on MS15, 23% of the embryos survived freezing to -196°C and warming to 23°C. When embryos were first matured in detached cobs and then dehydrated on MS15, survival was again observed after 5 days. The difference was that the time spent in each treatment was shorter. After one day on the detached cob followed by 4 days on MS15, 25% of the embryos survived, whereas after 2 days on the detached cob followed by 3 days on MS15, 27% of the embryos produced callus.

Vitrification Solution Toxicity

The solutions chosen were differentially toxic to maize immature embryos. A decrease in embryo survival was seen after longer incubation times, with VSY2 being the least toxic and TOWILL being the most toxic (Fig. 4.2). The addition of PBS amplified the toxicity. This is unfortunate since PBS increased the glass-forming tendency of VSY2 and PVS2. Towill's solution (1990) vitrified readily but was also fairly toxic to maize embryos. To see where the damage was occurring, embryos were incubated in VSY2+PBS for 15 min, unloaded and then incubated in TTC. Only the embryo axis produced a red color. The scutellum was white, indicating that the scutellum was more sensitive to the toxic effects of the vitrification solutions. This could also be seen by incubating freshly isolated embryos for various lengths of time in VSY2+PBS followed by unloading with 1.2M sucrose before TTC incubation. A progressive loss of scutellar viability was observed (Fig. 4.3A).

Abscisic acid was included in freeze-hardening experiments for two reasons. First, the addition of ABA prevented embryos from germinating. Embryos on MS15 enlarged and exhibited coleoptile elongation, indicating the onset of germination. Second, ABA has been shown to enhance freezing tolerance of plant tissues. After one week on MS15 with 50µM ABA the embryos were no longer sensitive to loading and unloading according to the TTC test and *in vitro* culture (Fig. 4.3B). The next step was to determine how long the embryos had to be on ABA for the scutella to develop resistance to loading and unloading. Embryos were therefore subjected to the TTC test after various lengths of incubation time on MS15 with ABA. Surprisingly, scutella survived a 30 min. exposure to VSY2 after only 24 hours on MS15 with ABA. The scutella were bright red and the embryo axes were intensely red. The scutellar intensity increased after two and three days of incubation on ABA.

Vitrification

Solutions made up according to the literature did not vitrify consistently. With the addition of PBS (1X), however, PVS2 and VSY2 vitrified much more consistently. Rapid plunging into LN was more effective than slowly lowering the tubes containing the solutions. The solutions also vitrified more consistently when Nunc cryotubes were used instead of Kimble glass tubes (Fig. 4.4A).

There was no survival of freshly isolated embryos after vitrification in PVS2 + PBS, VSY2 + PBS, and TOWILL according to *in vitro* culture. Survival was observed, however, for embryos vitrified in VSY2 + PBS after a 24 hour pretreatment on MS15. Survival was indicated by a positive reduction of TTC, which was apparent only in the embryo axis (Fig. 4.4B). Unfortunately, these embryos did not grow in culture. After a 24 hour pretreatment on MS15 containing 50µM ABA, however, both the scutellum and the embryo axis were able to reduce TTC and grow *in vitro* after vitrification and warming (Table 4.5). The scutellum remained small and white while the embryo axis enlarged and germinated (Fig. 4.5, and Fig. 4.6A). Roughly thirty percent of the embryos

germinated to produce plants using this approach. Hardening of the scutellum increased after 7 days pretreatment on ABA resulting in 95% of the embryos surviving vitrification and warming. Since not all of the embryos survived, different degrees of desiccation tolerance may have been present. This is observed in the growth of embryos after warming. Most of the embryos produce both roots and shoots, but some produce only one or the other (Fig. 4.5), possibly representing embryos with less desiccation tolerance. Germinated embryos were easily transferred to soil and grew to mature fertile plants (Fig. 4.6).

DISCUSSION

At 10-14 DAP maize embryos are desiccation sensitive (Delvallee et al., 1989). For this reason freshly isolated immature embryos did not survive constant rate cooling. Delvallee et al. (1989) concluded that the high moisture content of the embryos was the factor limiting survival after freezing, and that dehydration caused by ice formation is lethal at this stage. Even with addition of DMSO as a cryoprotectant maize immature embryos did not withstand freezing. One therefore must induce dehydration tolerance before maize immature embryos can be cryopreserved. Induction was accomplished by preculturing the embryos on various osmotica or by incubating detached cobs at 4°C. Preculture on osmotica apparently enabled the embryos to make the biochemical and developmental changes necessary to survive dehydration. These induced changes also enabled the embryos to survive the dehydration caused by freezing. The results show that preculture on MS15 does not adversely affect embryo development after being transferred back to MS6. Extensive dehydration on MS30, however, proved to have adverse effects on embryo development. Moderate dehydration (MS15) is therefore more suitable for inducing dehydration tolerance. The damage caused during dehydration by preculture on MS30 was exacerbated by freeze-induced dehydration; none of these embryos survived. Embryos precultured on MS30 did not survive either

slow or rapid freezing, suggesting that too much dehydration is not appropriate for embryos at this stage of development. Immature embryos therefore behave similar to recalcitrant seeds (Vertucci et al., 1991).

Moderate dehydration, was less damaging and induced more beneficial changes which enabled the embryos to survive dehydration incurred during freezing. Survival may be due to the lower water content in the dehydrated embryos or to intracellular changes that accompany desiccation stress. Changes in protein composition as well as membrane alterations during dehydration may be as important to survival as removal of water. Preculture on MS6 containing DMSO resulted in abnormal growth after transfer to MS6. Boucaud and Cambecedes (1988) showed that DMSO is toxic to imbibed seeds of maize, and suggested that propylene glycol together with 10% sucrose provided better cryoprotection. Since DMSO pretreated embryos did not survive freezing, it is likely that DMSO did not induce dehydration tolerance.

Both cold and osmotic pretreatments enabled maize immature embryos to survive freezing and thawing. After 5 days at 4°C immature embryos were isolated and cryopreserved. Of the embryos that survived only callus was observed. It appears that as the period of time spent maturing in the detached cob increases, so to does the likelihood of callus formation. Perhaps the chilled cob provided the embryos with a hormonal signal intended to prepare and enable them to survive chilling. After warming this signal may have been responsible for the production of callus. For example, endogenous auxin levels might increase, which may induce callus growth after warming. Alternatively, preculture at 4°C may induce tolerance to dehydration but does not initiate the removal of water from the embryos, resulting in a significant amount of extracellular ice during freezing which disrupts cellular connections. The callus production may simply be a wound response. Preculture by dehydration on MS15 alone resulted in cell conditioning and the removal of tissue water. Combining these two freeze-hardening methods had mixed results. Two days at 4°C followed by embryo isolation and 3 days on MS15 resulted in callusing of

27% of the embryos. Again these embryos may have been conditioned by hormonal signals in the chilled cob which resulted in callusing after freezing and thawing, or the presence of too much extracellular water resulting in wounding. Since alone 3 days on MS15 is not enough to induce freezing tolerance, significant tissue water may have been a factor. Four days on MS15 after one day at 4°C in the cob enabled embryos to survive freezing and thawing without callus formation. These embryos had the advantage of chill and dehydration induced conditioning. The short chill period and dehydration on MS15 together have enabled the shoot and root apices to develop. Perhaps the chill induced signal was too brief or that enough tissue water was removed to prevent callus formation after thawing.

Embryos freeze-hardened on MS15 for 5 days were sensitive to chilling, dehydration and cooling rate. When cooled to -5°C and warmed without ice formation viability of the root apex decreased by 20%. This damage may be caused by one or more of the injuries known to be associated with chilling. Among these are the reduction of metabolic rate, reduction of membrane fluidity, and the dehydration of proteins (Levitt, 1980). The differential sensitivity of the shoot and root apices to chilling may be due to differences acquired during preculture. Either the root apex is unable to fully acquire chilling tolerance or the preculture treatment was not long enough to allow for full chilling tolerance.

Embryos cooled to -40°C before warming to 25°C showed a significant loss of viability. Such a reduction is either due to dehydration or ice formation. A significant amount of ice may have accumulated outside or within the embryos by -40°C causing severe damage. Ice may have spread throughout the embryos beginning in the apoplasm. As the temperature decreased to -40°C more and more ice would have formed causing the extracellular fluid to become increasingly concentrated, resulting in cell dehydration. Excessive dehydration may have been responsible for the reduced viability when cooled to -40°C. Freeze-hardened embryos were likely more tolerant to this dehydration than fresh embryos. Preculture on MS15 would have removed some of the tissue

water and have added some sugars which would have played a colligative role in reducing the extracellular ice. Plunging embryos to -196°C from -40°C did not result in much more damage. It is likely that immersion of embryos in LN from -40°C produced rapid cooling and resulted in vitrification of the remaining fluids including the cell cytoplasm, thus preventing further ice damage and dehydration. Alternatively, all the freezable water may have been removed prior to plunging into LN. Embryos plunged from room temperature into LN had only root development after warming. Since the entire sample did not vitrify the cells of the root apex may have responded more rapidly to osmotic stress than those of the shoot apex. Again ice probably originated in the apoplasm, but at such a rapid cooling rate cells that could not respond osmotically to the changing extracellular osmotic pressure would have suffered intracellular freezing. It is probable that all of the shoot apices suffered from intracellular freezing whereas some root apices did not. Since roots are organs evolved to absorb water, root apices may have an inherent ability to respond rapidly to changing osmotic conditions. Once again it is evident that shoot and root apices are differentially sensitive to not only temperature stress but also to cooling rate.

The constant rate cooling method can be used to preserve maize immature embryos. Unfortunately, a long freeze-hardening period was needed prior to freezing. This may be a disadvantage if embryos are required at a specific stage of development. During freeze-hardening embryos may develop beyond the desired stage or may lose a desired characteristic. The initiation of embryogenic callus, for example, is possible only during a brief period of embryo development in grasses (Duncan et al., 1985). Grass embryos that have been cryopreserved therefore must retain their ability to produce callus after storage. The success obtained is promising since cooling rate and cryoprotectants were not optimized for immature embryos. Some basic knowledge was obtained for maize immature embryos during freezing. This knowledge may lead to improved constant rate cooling procedures resulting in reduced freeze-hardening pretreatments.

Vitrification is an alternative method of cryopreservation to constant rate cooling. Ice is avoided in this method by using concentrated solutions and fast cooling rates. The glass transition that occurs is considered virtually harmless. Problems with vitrification are finding a solution that is least toxic to the tissue and a cooling rate rapid enough to avoid ice formation. I selected three solutions from the literature for cryopreservation of immature embryos of maize. Two (PVS2 and TOWILL) had previously been used successfully to preserve plant cells (Towill, 1990; Uragami et al., 1989).

Solutions for vitrification often contain toxic concentrations of cryoprotectants. PVS2 is widely used in plant cryobiology and has been effective for storage of maize embryos (Table 4.5). VSY2 was the least toxic to the embryos and vitrified consistently. The most toxic solution (Towill) may have had a damaging concentration of ethylene glycol. Initially I thought that the toxic effects of the solutions were either chemical or osmotic toxicity. After using the TTC test, however, the toxicity was observed to be restricted in the scutellum. Since the embryo axis was relatively resistant to prolonged VSY2 exposure the toxicity was most likely not chemical in nature, but rather osmotic. The scutellum is, therefore, dehydration sensitive and is likely responsible for the recalcitrant nature of maize immature embryos to cryopreservation. During *in vitro* culture the embryo axis is separated from the nutritive medium by the scutellum. For healthy embryos the scutellum absorbs nutrients from the culture medium and transfers them to the developing embryonic axis. Since the scutellum is dehydration sensitive, the embryonic axis may become deprived of nutrients after dehydration stress and loss of scutellar activity.

Pretreatment on MS15 containing ABA promoted desiccation tolerance in scutella of immature embryos. This acquired tolerance enabled maize immature embryos to survive the loading and unloading of concentrated solutions required for vitrification. Abscisic acid has long been associated with dormancy and chilling tolerance in plants. ABA promotes dormancy in maize immature embryos (Hole et al., 1989). In addition ABA may act as a substitute for cold in

freeze-hardening by triggering the same genetic system (Reaney and Gusta, 1987). Endogenous levels of ABA increase during seed development in poikilohydrous (desiccation-tolerant) seeds and during cold acclimation. In fact ABA synthesis is essential for acquisition of chilling tolerance in maize seedlings (Anderson et al., 1994). ABA therefore acts to prepare plant cells for cold and the dehydration stress that accompanies freezing. Previous reports have shown that ABA induces dormancy in maize immature embryos. I have also observed a delay in germination of ABA treated embryos (data not shown). Since ABA treated immature embryos acquire dormancy and develop dehydration tolerance, ABA plays a key role in seed development. Exogenous application of ABA therefore initiates the premature expression of genes involved in desiccation tolerance and dormancy. Since the effects of ABA were concentrated in the scutellum, efforts should be made to elucidate the role of ABA in gene expression and development in scutellar cells of immature embryos.

Vitrification is an effective method for cryopreservation of maize immature embryos. With the application of a short freeze-hardening period on ABA the immature embryos become tolerant to dehydration and chilling. The short freeze-hardening period enable embryos to survive vitrification and warming. Freeze-hardening with ABA needs to be applied to constant rate cooling for a comparison of the two methods. Vitrification did however result in greater survival after freeze-hardening on MS15 compared to constant rate cooling. Collectively the addition of an ABA pretreatment and the application of salts during cooling enabled maize immature embryos to be cryopreserved using vitrification. This success will make immature embryos more readily available for study and will provide a means for the storage of *in vitro* produced embryos. As well, the ease and reduction of storage space make this technique a valuable tool in maize *in vitro* biology.

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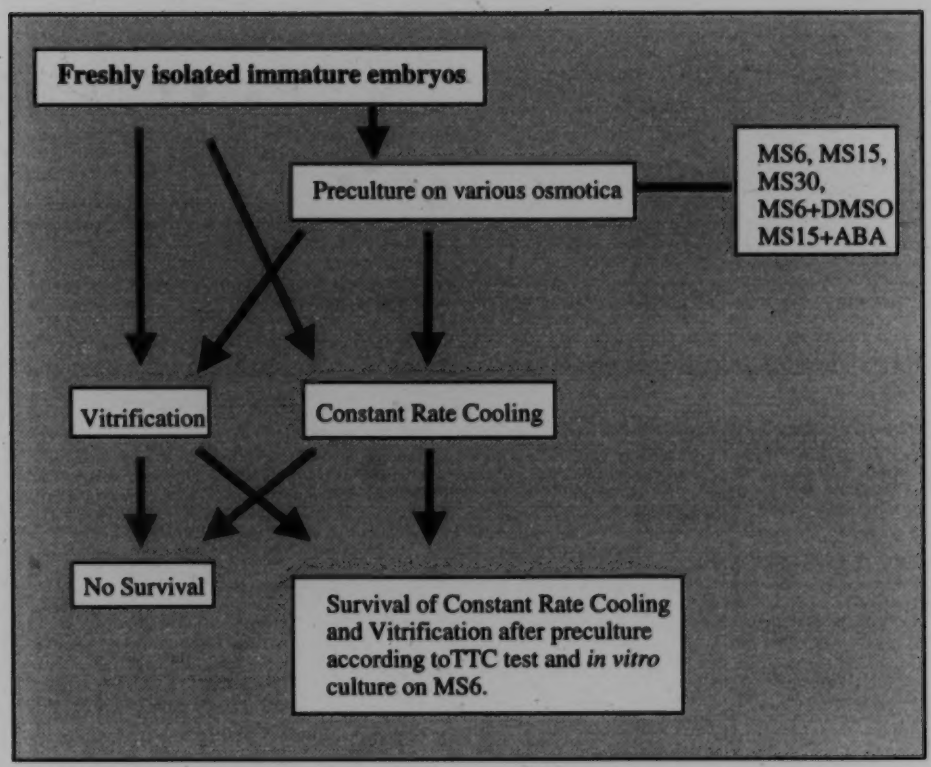

Fig. 4.1 Scheme for cryopreservation of maize immature embryos. Freshly isolated embryos could not survive Constant Rate Cooling or Vitrification. When embryos were precultured on various osmotica, however, survival was obtained to different extents.

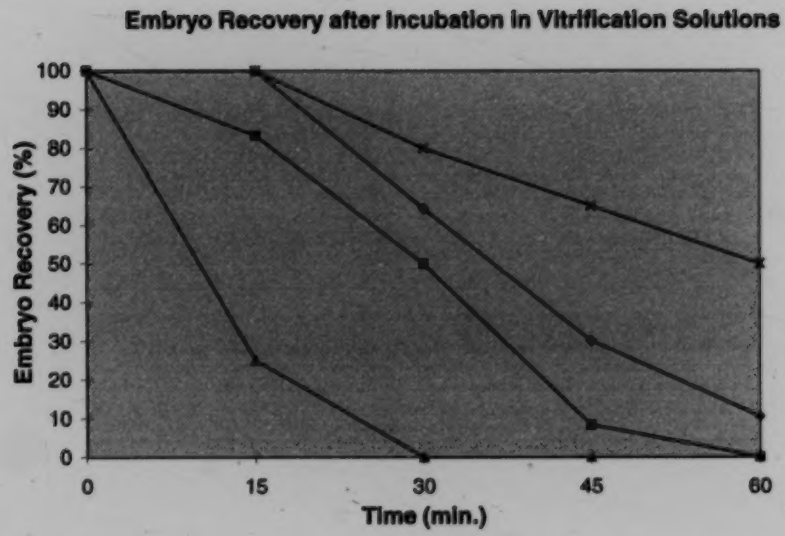

Fig. 4.2 Recovery of maize immature embryos after incubation in various vitrification solutions. Freshly isolated embryos were incubated in full strength solutions and then placed directly on MS6. Survival included either growth of callus, roots, or shoots based on a single experiment with 30 embryos per treatment.

Key: -▲- VSY2+PBS, -■- TOWILL, -◆- PVS2, -X- VSY2.

Fig. 4.3 Embryos were isolated at 13 DAP and incubated in VSY2 + PBS for various lengths of time. From left to right the embryos were incubated in VSY2 + PBS for 5, 10, 15, and 30 min before unloading with 1.2M sucrose and incubation in TTC overnight. A) Freshly isolated embryos. Note the progressive loss of scutellar viability. B) Embryos cultured on MS15 + ABA for one week prior to treatment. Note the tolerance of the scutella to VSY2 + PBS by the intense red color even after prolonged exposure. Bars = 2 mm.

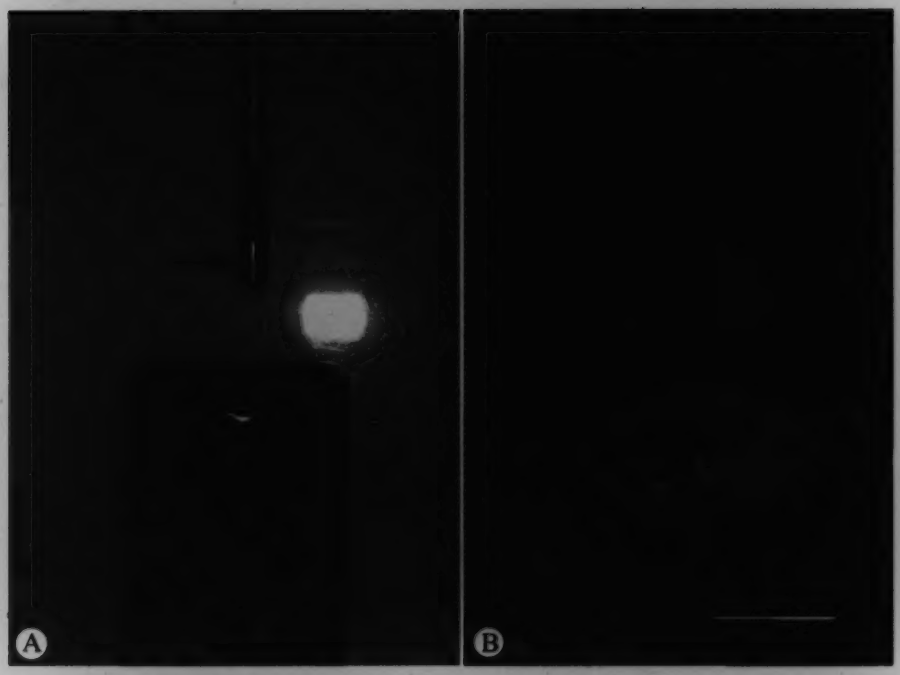

Fig. 4.4 VSY2 + PBS vitrifies consistently allowing embryo storage at -196°C. A) Plunging of Nunc tubes containing VSY2 + PBS results in vitrification. The arrow points to a Nunc cryotube containing vitrified VSY2 + PBS. The transparent nature of the solution indicates that no ice is present and that a glass transition has occurred. B) Thirteen DAP embryos survive vitrification in VSY2 + PBS after a 24 hour pretreatment on MS15. Although these embryos were unable to grow *in vitro* after vitrification their embryo axes were viable according to the TTC test. Note the red, viable embryo axes and apparently dead scutella. The arrow points to a crack that formed during cooling. Bar = 1 mm.

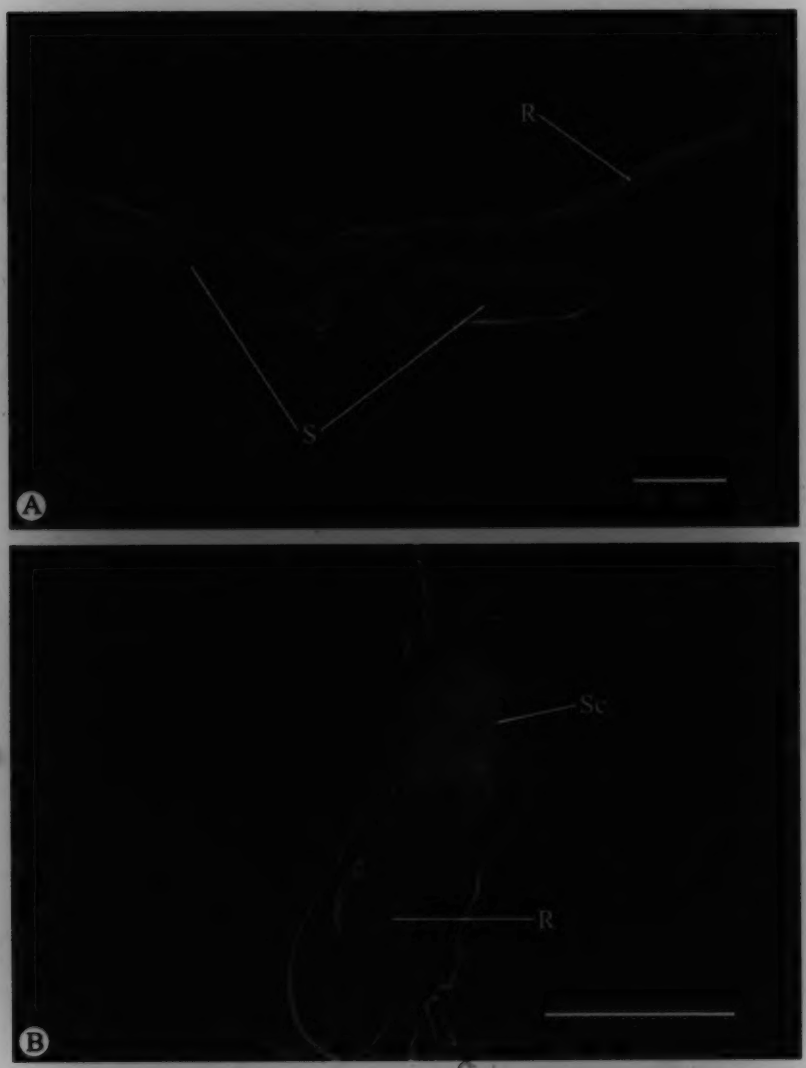

Fig. 4.5 Immature embryos survive vitrification. Survival depends on the tolerance of embryos to dehydration. A) Embryos with slight tolerance to dehydration rarely have complete survival. Instead survival of either roots or shoots is observed. The embryo on the left had both root and shoot survival whereas the embryo on the right had only shoot survival. B) Only the root apex was able to survive the dehydration for vitrification. Abbreviations: R, root; S, shoot; Sc, scutellum. Bars = 2 mm.

Fig. 4.6 Maize immature embryos survive vitrification and grow in culture to produce normal healthy plants after pretreatment on MS15 + ABA. A) After a 24 hour pretreatment on MS15 + ABA the embryo axis enlarges when placed on MS6 after vitrification in VSY2 + PBS. Bar = 2 mm. B) Embryos surviving the dehydration necessary for vitrification germinate on MS6 after vitrification and warming. Bar = 5 mm. C) Seedlings are transferred to soil and grow to produce fertile plants. *Abbreviations:* EA, embryo axis; R, root; Sc, scutellum; S, shoot.

Table 4.1 Composition of vitrification solutions in percent (w/v).

Component	VSY2	PVS2	TOWILL
Propylene glycol(PG)	10	—	—
Ethylene glycol(EG)	15.5	15	35
Dimethyl sulfoxide (DMSO)	20.5	15	7
Polyethylene glycol (PEG, 8000 MW)	6	—	10
Glycerol	—	30	—

Table 4.2 Growth of maize embryos after transfer from pretreatments to MS6.

Pretreatment	Germination (%)	Growth*
MS6	82.7±3.96	normal
MS6 + 5% DMSO	84.5±3.03	abnormal
MS15	95.4±1.63	normal
MS30	86.2±2.24	abnormal

* See text for description of growth.

Table 4.3 Survival of maize embryos after supercooling and various freezing treatments.

Pretreatment[1]	Supercooled to -5°C for 30min.	Slow cooled to -40°C (seeded at -10°C).	Slow cooled to -40°C followed by immersion in LN.	Rapid cooling by direct immersion in LN.
MS6 + 5%DMSO	ND	ND	0*	0
MS15	100s, 76.7±3.33r	29.2±1.73s,r	24.6±1.29s,r	0s, 19.5±6.96r
MS30	ND	ND	0	0

[1]Embryos were pretreated for 5 days at 25°C in the dark. *Values are in percent. *Abbreviations:* s, shoot growth; r, root growth; LN, liquid nitrogen; ND, not determined.

Table 4.4 Effect of combining pretreatments on the survival of maize embryos after freezing.

Days in cob at 4°C.	Days on MS15 at 25°C in the dark.	Survival after freezing. (in percent)
1-3	0	0
5	0	13.3±3.85c
0	1-3	0
0	5	24.6±1.29s,r
1	4	25.0±11.8s,r
2	3	26.7±7.70c

Abbreviations: c, callus; s, shoot growth; r, root growth.

Table 4.5 Growth of maize embryos on MS6 after vitrification in either VSY2+PBS or PVS2+PBS. Four different freeze-hardening treatments were applied.

Vitrification Solution	8 days on cob	7 days on MS15+ 50μm ABA	2 days on MS15+ 50μm ABA	1 day on MS15+ 50μm ABA
VSY2+PBS	ND	95.4±2.31	45.7±6.95	33.3±13.33
PVS2+PBS	96.7±3.33	95.1±2.48	ND	ND

CHAPTER 5

In Vitro Production and Long Term Storage of Maize Zygotic Embryos Derived from Isolated Embryo Sacs

INTRODUCTION

The growth of plant cells *in vitro* has revolutionized plant biology and the commercial plant industry. By controlling the extracellular environment we have learned about the *in vitro* behavior of cells, which has contributed to our understanding of their behavior *in vivo*, and has allow us to exploit them for commercial purposes. *In vitro* plant culture has contributed significantly to mass propagation, and has allowed numerous genetic transformation protocols to be developed (Vasil, 1988). As well, the totipotent nature of many somatic cells has stimulated an enormous interest in embryogenesis (Emons and Kieft, 1991). Recently advances in *in vitro* fertilization have directed some of that interest toward *in vitro* zygotic embryogenesis (Kranz and Lorz, 1993). Direct development from zygotes creates the potential for producing uniformly transformed plants which can then be mass propagated (Leduc et al., 1996).

Until recently maize embryo sacs containing zygotes were difficult to obtain and grow *in vitro*. Enzymatic and surgical dissection methods have allowed the current *in vitro* culture protocols (Campenot et al., 1992; Mol et al., 1995; Wagner et al., 1989), but these have their limitations (Leduc et al., 1995). Enzymatic digestion of ovular tissues results in the removal of nucellar cells which appear to be important for normal embryogenesis (Campenot et al., 1992; Leduc et al., 1995). Surgical dissection on the other hand is too labor intensive and often results in poor embryo sac visibility due to the surrounding nucellus. Recently, mechanical sectioning of ovaries has allowed large numbers of embryo sacs to be isolated with enough transparency to see individual cells (Chapter 2). These sections can be maintained *in vitro* on modified MS medium and develop without the addition of exogenous hormones. Embryogenesis occurs and mature plants can be obtained with ease. As *in vitro* production of plant embryos becomes more common a suitable method for dealing with large quantities of

embryos must be developed. Embryos will need to be stored for later use (Iriondo et al., 1992). Storage may be achieved using cryopreservation or, perhaps, by artificial seeds (Attree and Fowke, 1993; Bajaj, 1985).

Cryopreservation has been used for many years to preserve plant cells, and has many advantages (Kartha, 1985). Storage in LN prevents cellular decay and is relatively inexpensive. When seeds cannot be obtained or where seeds are recalcitrant, cryopreservation is often used to store germplasm (Bouchaud et al., 1991). For plant cells that can withstand dehydration the vitrification method is often applied (Fahy, 1988). This method employs rapid cooling by plunging samples into LN, thereby eliminating the need for a programmable freezer. A virtually harmless glass transition occurs which avoids the damage associated with ice.

Immature embryos isolated from maize and other grasses have been cryopreserved (Delvallee et al., 1989; Gnanapragasam and Vasil, 1992). Freeze-hardening is employed to lower embryo water content and to initiate cellular changes that allow the embryos to survive dehydration (Grout et al., 1983). Preculturing embryos on various cryoprotectants, or on osmotica help to promote dehydration and evoke freeze-hardiness (Delvallee et al., 1989). As well, preculture on media containing ABA has been demonstrated to increase the tolerance of tissues to dehydration and improve their survival after freezing (Reed, 1993; Zeevaart and Creelman, 1988). All of these freeze-hardening treatments prepare embryos for dehydration which occurs during ice formation or during loading of cryoprotectants prior to vitrification.

The present study utilized mechanical sectioning to obtain maize embryo sacs containing zygotes. Embryos were obtained by *in vitro* culture of isolated embryo sacs on MS media and were stored in LN using a vitrification method. After warming the embryos were germinated and grown to maturity. This is the first report of *in vitro* regeneration of plants from zygotes that includes an intermediate long term storage step.

MATERIAL AND METHODS

Plant Material

Plants of *Zea mays* L. cultivar CV129 were grown to maturity in growth rooms at the University of Alberta. At anthesis, pollen was collected by shaking tassels over an enamel tray and used immediately for hand pollination. Cobs were produced in a pollen-free environment by removing plants from pollen-producing rooms prior to silk emergence. The plants were emasculated, washed and placed in a pollen-free room for cob development. When emergent silks were between 6 to 10 cm in length the plants were removed from the pollen-free room and hand pollinated with fresh pollen. Cobs were subsequently bagged and the plants then placed in a growth chamber for fertilization to occur. Embryo sacs were isolated at least 16 hours after pollination.

Isolation of Embryo Sacs

Cobs were harvested and their husks and silks removed. Subsequent steps were carried out in a laminar air-flow hood. Three centimeter cob sections were surfaced sterilized for 10 min. in 70% ethanol followed by three rinses in sterilized distilled, deionized water. Ovaries were then removed and fastened to sterilized sectioning blocks using a fast acting adhesive (Quick Set 404, Locktite Corporation, Newington, CT). The blocks were then placed in the Vibratome (Technical Products International, Inc., St. Louis, MO) and the ovaries sectioned at 250-300μm. Sections containing intact embryo sacs were collected and placed in culture on various MS media.

In Vitro Culture of 1DAP Embryo Sacs

Embryo sacs were cultured *in vitro* on MS media and monitored for endosperm and embryo development. One DAP embryo sacs were placed on modified MS medium (Campenot et al., 1992) containing either high (15%) or low (6%) sucrose in the presence (0.1 mg/ml) or absence of the hormone BAP. Sections containing intact 1 DAP embryo sacs were placed on each of the selected media and monitored daily.

Embryo Rescue and Freeze-Hardening

When immature embryos developed from sections (between 2 and 3 weeks in culture) they were rescued and freeze-hardened prior to cryostorage. Hardening involved placing embryos on MS containing 10% sucrose (MS10) for two days followed by three or six days on MS containing 15% sucrose and 50μM ABA (MS15+ABA).

Loading Cryoprotectants & Cooling

One ml of VSY2 (10% propylene glycol, 15.5% ethylene glycol, 20.5% dimethyl sulfoxide, 6M polyethylene glycol) (Yang, 1992) containing PBS (1x) at pH 5.8 was added to each cryotube (Nunc, 1.8 ml). Loading involved incubating embryos in full strength VSY2+PBS at 25°C for 20 min. The solution was refreshed once prior to cooling. Tubes were gently shaken during loading to promote mixing and facilitate cryoprotectant penetration. Tube caps were securely tightened before the tubes were placed in aluminum holders and plunged rapidly into LN.

Vitrification Procedure

Cooling was rapid by direct immersion in LN. The solutions were considered vitrified when cooled to -196°C without ice formation. Once cooled the samples were stored in LN for at least 30 minutes.

Warming & Unloading Cryoprotectants

Rapid warming was achieved by placing the samples into a 45°C circulating water bath. The tubes were held in the water bath for 1 min. before removal of VSY2+PBS. After warming embryos were either placed directly on MS medium containing 6% sucrose (MS6) or first diluted by adding one ml of unloading solution (1.2M sucrose with PBS (1X) at a pH of 5.8) to each tube. After 10 min. of unloading the tubes were swirled and emptied into a sterile petri dish. Embryos were then placed embryo axis upward on MS6 culture medium. Viability was assessed by growth *in vitro*.

RESULTS AND DISCUSSION

Vibratome sectioning yielded embryo sacs in slabs of nucellus, providing embryo sacs with structural support as well as apparent growth stimuli (Campenot et al., 1992). Embryo sacs could be observed from at least one surface (Fig. 5.1A), and viability was not sacrificed since the embryo sacs were able to develop normally after sectioning. Embryo sacs isolated using digestive enzymes, however, lose their viability the longer they are exposed to digestive enzymes (Leduc et al., 1995). Vibratome-isolation of embryo sacs is therefore more practical since access to embryo sacs is accomplished without sacrificing viability. The sections can be grasped using forceps without injury, making media transfers easy to perform. Even with the ovary wall removed, the nucellus provided a "natural incubator" for embryo sac development.

Endosperm growth preceded embryo formation. The endosperm grew into a spherical shape with an embryo developing at or near the surface toward the micropylar end (Fig. 5.1B). The ovary wall grew significantly on MS6 and tended to push the nucellus away from the media (Fig. 5.2A). For this reason the ovary wall was removed from the sections prior to *in vitro* culture (Fig. 5.2B,C). Embryo sacs cultured on MS6 exhibited extensive endosperm growth (Fig. 5.1B). As well, embryos produced on MS6 tended to develop into callus when cultured in contact with the endosperm, making embryo rescue essential. Perhaps the endosperm was a source of auxin which stimulated embryos to form callus. Lur and Setter (1993) reported a rise in endogenous auxin levels beginning at 9 DAP in maize endosperm *in vivo*. A similar rise *in vitro* may adversely affect embryo development, and even cause callus formation. Alternatively the embryos may have been induced to form callus by malnutrition caused by the proliferating callus (Chang and Neuffer, 1994). Sections cultured on MS15 however, did not yield extensive endosperm growth and embryo development into callus was not a problem. Therefore, rescuing these embryos was not essential.

Embryos were obtained from MS6, MS6+BAP, and MS15+BAP (Table 5.1). Remarkably, embryos developed from MS without addition of hormones. This result supports the hypothesis that the nucellus is beneficial for stimulating zygotic embryogenesis. Even a thin slab of nucellus promoted and maintained embryo development when nutrients were provided. The zygotic embryos proceeded directly to plants without an intermediate callus stage, which could have introduced somaclonal variation.

Coleoptilar to stage 1 embryos appeared after roughly 10 days in culture, and were rescued and placed with their scutella down on MS10 (Fig. 5.3A). During incubation on MS10 the embryos enlarged slightly and became less transparent. This step was included to wean the embryos from the endosperm and nucellus and to promote further development since the MS medium contained a relatively high sucrose concentration. After two days on MS10 the embryos were transferred to MS15+ABA to promote desiccation tolerance. Endogenous ABA levels are known to rise in plant tissues subjected to chilling and dehydration stress (Reaney and Gusta, 1987). This rise in endogenous ABA levels is thought to initiate cellular changes that allow the plant tissue to survive stressful conditions (Anderson et al., 1994). Similarly, exogenous ABA applications are useful for preparing plant tissues to survive cryopreservation (Reed, 1993). In the current study, *in vitro* produced embryos were incubated on MS15+ABA for three and six days. Three days on ABA provided *in vivo* produced immature embryos with a high degree of dehydration tolerance (Chapter 4), and therefore was considered adequate for *in vitro* produced embryos. Six days on ABA was included to ensure that adequate tolerance to dehydration had been reached, since *in vitro* produced embryos may not have behaved the same as *in vivo* produced embryos. During incubation on ABA the embryos enlarged but did not germinate (Fig. 5.3A). Together MS15+ABA maintained the embryos in a developmental mode and prevented germination, as seen for older maize embryos (Oishi and Bewley, 1992).

After hardening on ABA the embryos were tolerant to dehydration and survived cooling to -196°C (Table 5.2). Survival (presence of roots, shoots or callus) was approximately 90% after vitrification for embryos produced on the three different media after 3 days on ABA. There was a slight increase in survival (more roots and shoots and less callus) of embryos hardened for 6 days on ABA compared to 3 days, indicating a progressive increase in dehydration tolerance with exposure to ABA. Either roots, shoots or callus grew from the embryos. Embryos initiated on MS15+BAP had less callus and more root and shoot growth after vitrification when compared with embryos initiated on MS6. The embryos developing on MS15 may have acquired a higher degree of desiccation tolerance even prior to ABA treatment, which may not have occurred when embryos were developed on MS6. The unloading step slightly increased embryo survival for embryos generated on MS containing 6% sucrose (Table 5.2). These embryos are therefore more sensitive to the toxic effects of VSY2+PBS, and likely less desiccation tolerant. Overall survival was higher than that seen for *in vivo* produced embryos treated similarly (Chapter 4). Perhaps the *in vitro* environment had enabled the embryos to be more self-sufficient, thus preparing them for dehydration stress. Embryos produced *in vivo*, however, are more dependent on and protected by the maternal environment and therefore less tolerant to stress. Embryos germinated from the three treatments and grew to produce normal, healthy plants (Fig. 5.3B,C). The *in vitro* environment and storage in LN therefore did not cause significant damage.

This is the first report of embryo development and storage entirely *in vitro*. It establishes the point that plant reproduction can easily be manipulated from the initiation of zygotic embryogenesis to the *in vitro* development of plants. Nearly all developmental stages can be controlled and arrested by storage in LN. This is an important factor for germplasm repositories and would be convenient for labs handling large quantities of embryos.

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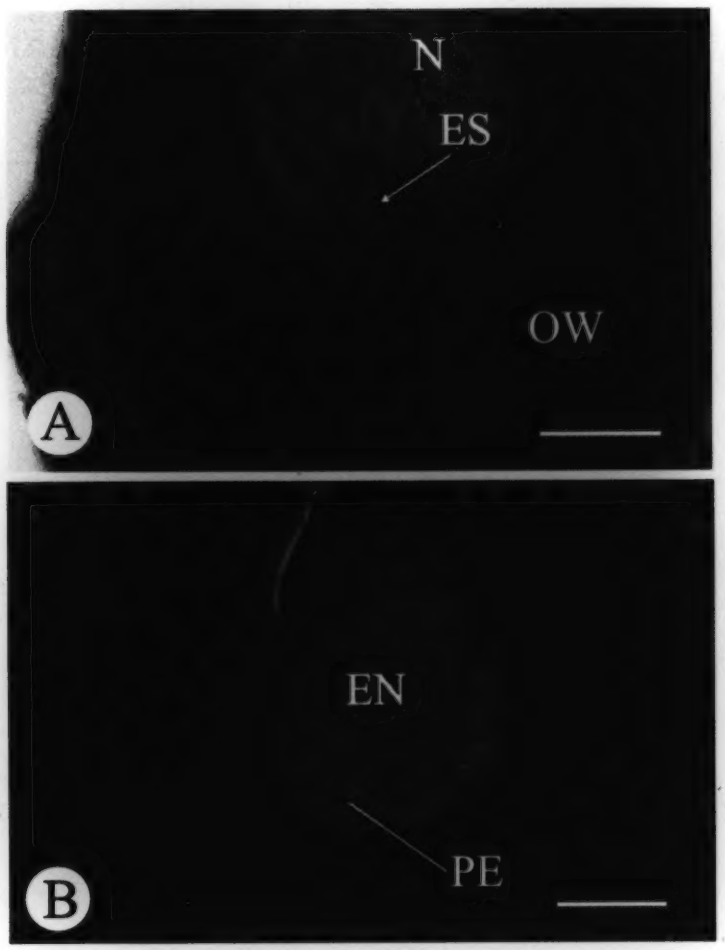

Fig. 5.1 Isolated embryo sacs containing zygotes produce embryos *in vitro*. A) Vibratome sections provide access to embryo sacs as well as supportive tissue to assist development. Depending on the quality of the section, individual embryo sac cells can be seen. In this section the antipodals, central cell, and egg apparatus are visible. Bar = 500 μm. B) When cultured *in vitro* on MS6 with the ovary wall removed, the endosperm grows into a large sphere and an embryo develops from the zygote usually near the surface. Bar = 1 mm. *Abbreviations:* ES, embryo sac; EN, endosperm; N, nucellus; OW, ovary wall; PE, proembryo.

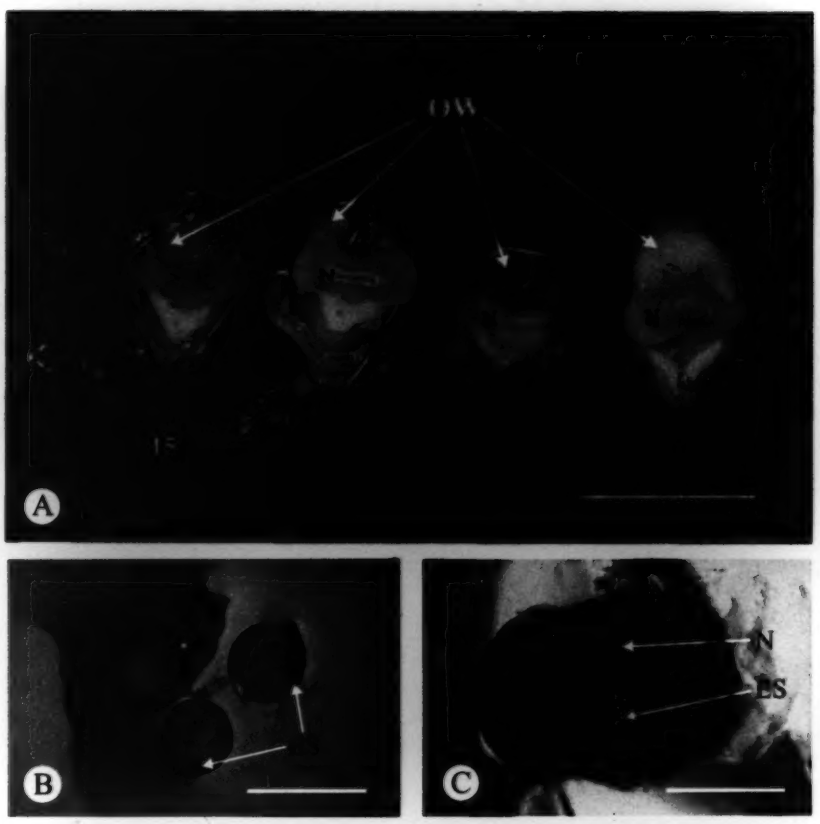

Fig. 5.2 Growth of Vibratome-isolated sections on MS6 resulted in extensive ovary wall growth leading to removal of nucellus from the culture medium. For this reason ovary walls were removed prior to *in vitro* culture on MS6. A) Sections in culture on MS6 and MS15. Note the more extensive growth of ovary wall and nucellus on MS6. The nucellus is forced away from the medium on MS6 compared to nucellus on MS15. Bar = 5 mm. B) Ovary wall is removed with ease leaving the embryo sac surrounded by nucellus. Two of these freshly isolated embryo sacs have had their ovary walls removed. Bar = 2 mm. C) During *in vitro* culture on MS6 endosperm growth soon becomes apparent. Bar = 1 mm. *Abbreviations:* 6, sections cultured on MS6; 15, sections cultured on MS15; *, ovary wall; ES, embryo sac; OW, ovary wall; N, nucellus.

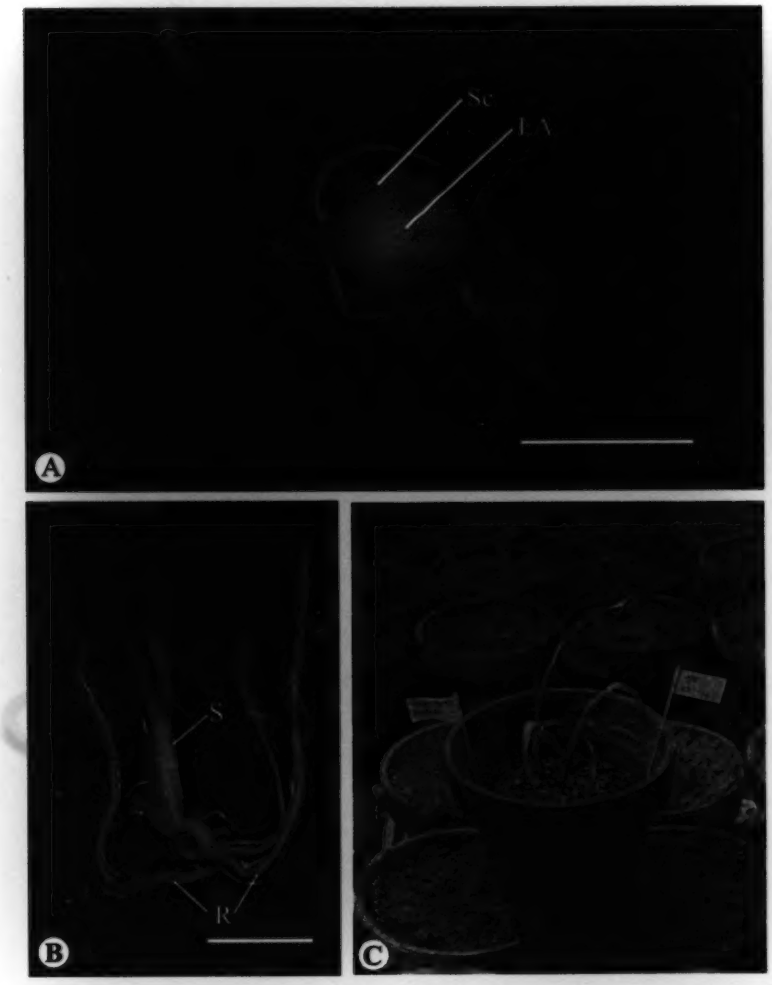

Fig. 5.3 Embryos produced *in vitro* survived vitrification and germinated to produce normal, healthy plants. A) Embryos were rescued and placed with their scutella down on MS10 followed by MS15 + ABA to develop dehydration tolerance. This embryo was photographed after 3 days on MS15 + ABA. Bar = 1 mm. B) After vitrification embryos were placed in culture on MS3 to promote germination. This embryo has germinated to produce a seedling with normal roots and shoots. Bar = 5 mm. C) Once a significant amount of roots have formed the seedlings are transferred to soil. These seedlings have been in soil for two weeks. *Abbreviations:* EA, embryo axis; R, roots; sincerely, shoot, Sc, scutellum.

Table 5.1 Growth[1] of embryos and endosperm from partially isolated embryo sacs cultured *in vitro* on various MS media.

Culture Medium	Endosperm Growth	Embryo Growth
MS6*	79.3±0.74	55.6±8.01
MS6+*	89.9±2.10	69.5±1.76
MS15+	81.4±6.44	75.0±3.61

[1]Values are means ± standard error calculated from triplicates of thirty embryos per treatment.

* Sections had their ovary walls removed prior to *in vitro* culture.

Table 5.2 Survival[1] of embryos to vitrification indicated by growth of either callus, roots or shoots.

Culture Medium	No wash[2] after warming	Washed after warming
MS6*[3]	75.9±4.09	81.6±5.43
MS6*	80.9±4.61	90.3±5.78
MS6+*	82.6±3.76	90.0±5.77
MS15+	90.0±5.77	91.1±4.84

[1]Values are means ± standard error calculated from triplicates of thirty embryos per treatment.
[2]Wash consisted of a 20 min. incubation in 1.2M sucrose prior to *in vitro* culture.
[3]On MS15 + ABA for 3 days, otherwise for 6 days.
*Sections had their ovary walls removed prior to *in vitro* culture.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

Before this study, maize embryo sacs were isolated using either microdissection or digestive enzymes (Campenot et al., 1992; Leduc et al., 1995). Both of these methods have disadvantages including either production of embryo sacs with poor transparency or embryo sacs with reduced regenerative ability. For these reasons I developed an alternative method for isolating maize embryo sacs which could allow *in vitro* fertilization and provide a simplified procedure for regenerating plants. A mechanical sectioning procedure was developed which can remove living embryo sacs from maternal protection with more precision, accuracy, and speed than surgical microdissection. Partially isolated embryo sacs resulted which were capable of regenerating plants using very simplistic *in vitro* culture procedures. These embryo sacs were contained in slabs of nucellus which may have been responsible for their regenerative success. The beauty of this isolation method is that embryo sac cells are visible and plant regeneration is direct and simple. As well, the isolation procedure is relatively easy to perform with minimal problems of manipulation.

There are many immediate applications of this invention, including genetic engineering and the potential for basic research into events surrounding fertilization. Since embryo sacs are intact and regenerate plants at rates much superior to previous methods, they can be used immediately for studies on embryogenesis and endosperm development. As well, future success with *in vitro* fertilization using unfertilized embryo sacs isolated with this method may also have high regeneration potential. Exploitation of these partially isolated embryo sacs is imminent. The immediate application is for genetic engineering either for purposes of studying genes and development or for producing plants with improved or added traits (Lindsey, 1992). As well genetic engineering could lead to production of novel products including plastics or vaccines. These potential applications in basic science and the commercial industry help stress

the importance and significance of mechanically isolating embryo sacs using Vibratome sectioning.

Chapter 3 discussed some interesting characteristics of maize embryo sacs and embryos in culture. Embryo sacs isolated at 5 DAP showed poor regenerative potential. This was an important result since it indicated that maternal tissues are important for stimulating embryogenesis. The fact that Vibratome-isolated embryo sacs at 1 DAP could regenerate plants with ease and 5 DAP embryo sacs completely isolated could not, indicates that the remaining maternal tissue is responsible for the success of Vibratome-isolated embryo sacs at 1 DAP. The nucellus plays an important role since embryogenesis occurs from 1 DAP embryo sacs with only the nucellus present (Campenot et al., 1992). Even with the presence of BAP, 5 DAP embryo sacs grew unsuccessfully in culture indicating that more than just this hormone is required for embryogenesis.

The results from *in vitro* culture of immature embryos revealed some interesting results as well. Embryos responded to different osmolality by either germinating or maturing before germination. Lower osmolality promoted embryo germination, whereas higher osmolality maintained embryos in a developmental mode (Xu et al., 1990). Very high osmolality proved to be detrimental to development, indicating that maize immature embryos are desiccation sensitive (Delvallee et al., 1989). A short dehydration period was effective at promoting germination, indicating a switch to germination mode (Kermode and Bewley, 1989). This observation is interesting since it has never been demonstrated for such young embryos and since it indicates that germination genes can be activated early in development.

With all the success in maize reproductive biology a method for storing *in vitro* produced embryos was in demand. Past attempts at cryopreserving maize embryos were largely unsuccessful, since long freeze-hardening procedures were required (Delvallee et al., 1989). I therefore attempted to make improvements on existing methods using vitrification. Hardening with ABA and optimizing vitrification resulted in a simple yet useful method of preserving maize

embryos. I then successfully applied this method to embryos produced *in vitro* from zygotes. These embryos were preserved with even greater success. The difficulty in cryopreserving maize immature embryos is due to their desiccation insensitivity. The TTC test proved that this sensitivity may be localized in the scutellum, indicating that the embryo axis is not desiccation sensitive. This observation needs to be studied in more detail and in reference to the germination switch result. Since the scutellum is the tissue most sensitive to dehydration then perhaps it is the site for recognition of dehydration as well, that switches embryos to germination mode after premature desiccation.

This study has generated several techniques which have potential benefits for maize *in vitro* biology. The techniques have commercial importance and were created out of demand in a rapidly growing field. Vibratome isolation of embryo sacs will contribution to maize biology since there are many advantages over other methods used to isolate embryo sacs. The results presented on *in vitro* culture bring us one step closer to understanding the behavior of maize embryo sacs and zygotic embryos. Similarly successful cryopreservation of maize embryos makes it easier to manipulate maize embryos *in vitro*. The knowledge and techniques developed in this study are important contribution to maize biology. It is my hope that these techniques and knowledge presented be applied to other species as well.

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